# Molecular Mechanisms of Acinar-to-Ductal Metaplasia Formation During Pancreatitis

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# 1. Summary

Pancreatitis is a severe inflammation of the pancreas and a major risk factor for developing pancreatic ductal adenocarcinoma, a human malignancy associated with a 5-year survival rate of less than 5%. A key step in the malignant transformation is the formation of acinar-to-ductal metaplasia (ADM), where acinar cells de-differentiate into a progenitor state characterized by the appearance of tubular complexes. Importantly, a transient ADM formation and concomitant acinar cell proliferation has also been observed during pancreatitis both in humans and rodents, suggesting that the plasticity of acinar cells and their ability to de-differentiate into progenitor cells are crucial processes for pancreas regeneration. The mechanisms behind proliferation of acinar cells and metaplastic processes remain elusive because physiological factors that drive their regulatory network have not been fully identified. In this research project we wanted to investigate mitotic and anti-mitotic factors that contribute to acinar cell proliferation and ADM formation during pancreatitis.

In the search for key molecular events that regulate ADM, we analysed the contribution of the cell cycle regulatory molecule $p21^{waf1/cip1}$ in initiating ADM and acinar cell proliferation during pancreatitis. By using a genetically engineered mouse strain deficient in p21, we could show that loss of p21 increased the extent of ADM formation without altering the ability of acinar cells to replicate. These data revealed that p21 regulates acinar cell de-differentiation independently from cell cycle modulation. Importantly, loss of p21 was accompanied by increased expression of β-catenin and development of senescence and in many ways both of these factors can contribute to ADM formation. Thus, our results indicate that p21 acts as a molecular switch limiting senescence and ADM formation.

In the second project we investigated the importance of extracellular factors in ADM formation. TGF-β signalling plays an essential role in tissue repair and we analysed the contribution of TGF-β signalling in acinar cell proliferation and ADM formation. To address these questions we generated transgenic mice lacking TGF-β receptor II (TGF-β RII) in pancreatic epithelial cells. Our mouse model showed that TGF-β signalling is necessary to limit ADM formation, most likely via crosstalk with EGFR signalling. Moreover, the data also revealed a weak anti-mitotic activity of TGF-β in acinar cells that is elicited by controlling the expression of the cell cycle regulator p16. Collectively, TGF-β signalling restrains both ADM formation and acinar cell proliferation, indicating its dual role during pancreatitis.

In the third project we investigated the mitogenic effects of serotonin, a well-known stimulator of cell proliferation, in ADM formation and acinar cell proliferation. More specifically, by using a genetically engineered mouse strain deficient in peripheral serotonin, we showed that the absence of serotonin delayed ADM formation and elicited a minor reduction of acinar cell proliferation. We proposed a possible mechanism of serotonin mediated ADM formation by

showing that serotonin stimulated acinar cell secretion and progenitor gene expression both processes being a pre-requisite for ADM formation. Our findings indicate that serotonin promotes de-differentiation of acinar cells and ADM formation following pancreatitis.

**Significance of the project:**

The results of this research indicate the contribution of pro- and anti-proliferative molecules in ADM formation and acinar cell proliferation during pancreatitis. It has been previously demonstrated that p21, TGF-β and serotonin regulate cell proliferation in different tissues. However, our analysis showed unique roles of these three molecules in pancreatic pathology where they regulate more ADM formation than cell proliferation. The characterization of this regulatory mechanism in pancreatic injury advanced our understanding of the control of pancreatic regeneration. However, still a lot of work needs to be done to fully characterize the regulatory network of ADM and pancreatic regeneration in order to reveal novel therapeutic strategies to block ADM progression towards pancreatic cancer.

# 2. Zusammenfassung

Pankreatitis ist eine entzündliche Erkrankung der Bauchspeicheldrüse. Sie erhöht die Wahrscheinlichkeit an Pankreaskrebs zu erkranken, bei dem die 5-Jahre Überlebensrate weniger als 5% ist. Ein kritischer Prozess für Entstehung von Pankreaskrebs ist die Bildung von Azinus-zu-Dukt Metaplasien (ADM). ADM beinhaltet charakteristischerweise, eine Dedifferenzierung zu Vorläuferzellen und schliesslich die Ausbildung von tubulären Komplexen. Sowohl in Menschen wie auch in Nagetieren wurden während der Pankreatitis vorübergehende ADM beobachtet, einhergehend mit einer erhöhten Proliferation von Azinuszellen, sowie einer Proliferation in den ADM selbst. Dieser Prozess deutet auf eine Plastizität der Azinuszellen hin, und spielt damit eine wichtige Rolle bei der Regeneration des Pankreas. Die genauen Mechanismen, die für die azinäre Proliferation und die metaplastischen Veränderungen verantwortlich sind, sind noch unklar, da die Faktoren noch nicht komplett identifiziert wurden. In dieser Studie wollten wir pro- und anti-mitotischen Faktoren charakterisieren, die Proliferation und ADM kontrollieren.

Daher wurde auch die Funktion des Zellzyklus Inhibitors $p21^{waf1/cip1}$ während der Pankreatitis untersucht. Zur Untersuchung nutzten wir genetisch modifizierte Mäuse, denen das Gen für $p21^{waf1/cip1}$ fehlt. Diese Mäuse zeigten eine erhöhte Bildung von ADM Beeinträchtigung der Zellproliferation. Diese Ergebnisse lassen darauf schliessen, dass p21 die azinäre Dedifferenzierung reguliert, ohne den Zellzyklus zu modulieren. Ausserdem geht der Verlust von $p21^{waf1/cip1}$ mit einer Erhöhung der Expression von β-Catenin einher, sowie einer azinären Zell-Seneszenz. Beide Faktoren scheinen die ADM Bildung zu beeinflussen. Diese Resultate implizieren, dass $p21^{waf1/cip1}$ ein molekularer Schalter bei der Seneszenz und ADM Bildung ist.

In einer zweiten Studie untersuchten wir extrazelluläre Faktoren, die zur Bildung von ADM beitragen. TGF-β spielt eine wichtige Rolle in der Wundheilung. Deshalb untersuchten wir die Signalkaskade in azinären Zellen und ADM, die durch TGF-β ausgelöst wird. Für diese Untersuchungen wurden transgene Mäuse hergestellt, deren TGF-β Rezeptor (TGF-β RII) selektiv im Pankreasepithelium fehlt. Dieses Mausmodell hat gezeigt, dass TGF-β die Bildung von ADM inhibiert, wahrscheinlich über die Aktivierung des EGF-Rezeptors. Ausserdem wurde eine anti-mitotische Aktivität von TGF-β in Azinuszellen beobachtet. Dabei wird angenommen das p16, ein Zellzyklusinhibitor, aktiviert wird. Zusammenfassend konnten wir eine Doppelrolle von TGF-β in der Regulation von ADM, sowie eine Inhibition der azinären Proliferation im Pankreas feststellen.

Im dritten Projekt untersuchten wir die Funktion von Serotonin in der ADM Bildung und azinären Proliferation. Serotonin ist ein bekannter mitotischer Faktor. Zur Untersuchung verwendeten wir eine transgene Maus, die kein peripheres Serotonin produzieren kann. In

diesem Modell war die ADM Bildung verzögert und die azinäre Proliferation leicht reduziert. Unsere Daten deuten darauf hin, dass Serotonin an der Sekretion sowie der Rekrutierung von Vorläuferzellen beteiligt ist. Beide Prozesse sind Voraussetzung für die Bildung von ADM. Wir schliessen daraus, dass Serotonin die Dedifferenzierung von azinären Zellen und die Bildung von ADM während der Pankreatitis fördert.

**Signifikanz des Projektes**

Die Resultate unserer Untersuchungen unterstreichen die Bedeutung von pro- und anti-mitotischen Faktoren bei der Bildung von ADM sowie der azinären Proliferation bei der Pankreatitis. Es war bekannt das p21, TGF-β und Serotonin die Proliferation in verschiedenen Geweben kontrollieren. Unsere Analysen der pathologische Veränderungen im Pankreas konnten nun zeigen, dass die Kontrolle der ADM Bildung durch diese Faktoren eine neuartige Funktion darstellt. Die Aufklärung dieser Mechanismen vertieft unser Verständnis der pathophysiologischen Veränderungen nach einem Gewebeschaden und offenbart neue mechanistische Aspekte der Pankreasregeneration. Nichtsdestotrotz, sind weitere Untersuchungen nötig, um die Regulation der Bildung von ADM während der pankreatischen Regeneration zu verstehen und allfällige therapeutische Ansätze aufzuzeigen.

# 3. Introduction

## 3.1 Pancreas

*Anatomy*

The pancreas is a glandular organ in the digestive and endocrine systems of vertebrates. It is the organ that probably most people have never thought about, but it can be felt after overeating when a feeling of vague and localised visceral pain arises. The pancreas lies in the abdomen behind the stomach and is surrounded by the duodenum. Anatomically, it is divided into head, where the greatest mass of the organ is concentrated, body and tail. The head of the pancreas is connected to the duodenum while the body and the tail angle distinctly as they approach the spleen. The pancreas secretes pancreatic juice, a thick, colourless, alkaline fluid containing all pancreatic enzymes. The main channel for pancreatic juice drainage is the main pancreatic duct. It begins in the tail, runs through the body and ends in the head, along the way connecting with many secondary ducts. Also the common bile duct passes through the head of the pancreas and joins the main pancreatic duct at entering the duodenum (see Figure 1).

*The pancreas as a double gland*

The pancreas is a secretory organ which contains an endocrine and exocrine tissue. The endocrine part constitutes ~5% of the organ and the exocrine part nearly 95% of the pancreatic mass. The endocrine part of the organ is embedded in the exocrine tissue and contains $\beta$, $\alpha$, $\delta$, PP and $\varepsilon$ cells that produce and release hormones like insulin, glucagon and somatostatin into the blood stream and control the metabolism of carbohydrates. The exocrine part consists of acinar and ductal cells. Acinar cells synthesize digestive enzymes and store them in zymogen granules. Ductal cells not only form pancreatic ducts but also secrete bicarbonates, ions and fluids that are necessary for draining zymogens into the duodenum where they are activated and participate in the carbohydrate, protein and lipid break down. Importantly, bicarbonates are required for stomach acid neutralisation at the entrance to the intestine in order to protect the intestine from acidity. Acinar cell digestive enzymes and ductal cell bicarbonates, ions and fluids form the pancreatic juice, the production of which can reach 1.5L per day.

The pancreatic juice is drained through a complex network of ducts. Pancreatic ducts can be subdivided into three major groups according to their location. The first group, referred to as the intralobular ducts, occurs within the lobules and drains pancreatic juice from acini. The second group, consisting of interlobular ducts, occur in the interlobular connective tissue together with the major branches of vessels and nerves. The interlobular ducts then drain into the third duct group, the main pancreatic duct, which transverses the pancreas to the duodenum.

Secreted pancreatic proteins tend to form aggregates and a control of the pancreatic juice viscosity by bicarbonate, ion and fluid secretion is of a paramount importance to maintain the protein flow along the ducts.

*Vasculature*

The pancreas is permeated by blood vessels supplying the gland and this dense vasculature can become an utmost problem causing massive organ bleeding in case of pancreatic injuries or diseases. The pancreas receives the arterial blood supply from two arteries: the celiac and the superior mesenteric. Around 20% of the intrapancreatic blood flow is directed towards the islets where released hormones drain straight into the blood stream.

*Innervation*

The pancreas is waved by a dense network of neurons. Depending on the severity of pancreatic diseases, 80-95% of patients experience pain, which is the most common reason for searching medical help. [2] The pancreas is innervated by parasympathetic and sympathetic nervous systems and both types of nerve fibres mediate pain in the organ. The parasympathetic innervation originating from the vagus nerve also stimulates enzyme secretion.

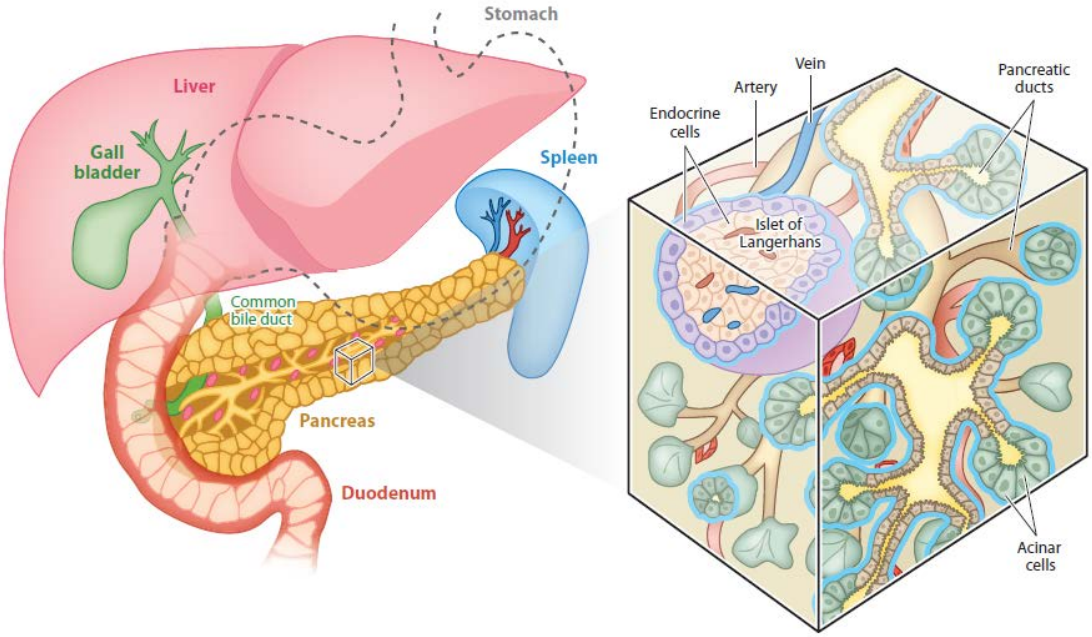


**Figure 1: Adult pancreas anatomy and structure. (Picture is taken from [1])**

*Biology of exocrine cells*

The main enzyme-secreting units in the exocrine pancreas are acini, a round structure composed of pyramidal-shaped acinar cells bordering a common luminal space, which is connected with a ductal system. The acinar cell of the pancreas is polarized: the basal pole of the cell is mainly occupied by the endoplasmic reticulum and the apical pole by the Golgi complex and the zymogen granules. Zymogens are inactive enzymes synthesized and folded

in the endoplasmic reticulum, later sorted, packed and prepared for secretion in the Golgi complex. Interestingly, an acinar cell produces and secretes more proteins than any other adult cell. These proteins fall into three major categories of digestive enzymes: α-amylase, lipase and proteases, which are responsible for the hydrolysis of carbohydrates, fats and proteins, respectively. Secretion of digestive enzymes is stimulated by various hormones and by the parasympathic innervation of the pancreas. Secretin, cholecystokinin (CCK), gastrin releasing peptide (GRP), substance P, vasoactive intestinal peptide (VIP) and acetylcholine are mediators of enzyme secretion. After binding to their corresponding acinar receptors of the G-protein coupled family, these hormones trigger the process of receptor-mediated secretion.

*Acinar and ductal cell development*

The pancreas arises from the ventral and dorsal foregut epithelium by branching and forming multi-layered, stratified epithelium. This early pancreatic epithelium contains multipotent pancreatic progenitor cells with the potential to give rise to all pancreatic cell types (see Figure 2). The 'tips' of the branching epithelium contain progenitor cells, while the 'trunk' region give rise to islet and ductal cells. At day e13.5 'tip' cells lose their multipotency and become acinar progenitor cells. After this period pancreatic cells undergo further growth and development. Developmental programs that are active in progenitor cells during embryonic development get activated once more during pancreatic injury, thus understanding the physiological molecular processes occurring during the development of the pancreas will help us understand better the pathological ones in the adult organ. [3]

Acinar cell embryonic development is regulated by multiple mechanisms: on one hand by transcription factors that drive pancreas formation and on the other hand, by extrinsic signals and cell-cell interactions. Acinar cells derive from multipotent progenitors that co-express the homeobox protein PDX1, the Sry-box protein SOX9 and Ptf1a, a basic helix-loop-helix (bHLH) pancreas specific transcription factor active during embryogenesis which is later maintained only in adult acinar cells. In the absence of Ptf1a, acinar cells fail to develop and one reasons is that Ptf1a regulates the expression of acinar specific genes, including digestive enzymes like amylase and lipase. Acinar cell development is guided also by many other embryonic signals. For example, secreted pancreatic mesenchymal factors FGF10 and follistatin promote acinar cell specification. Notch signalling can repress acinar cell fate, while, Wnt signalling promotes progenitor and acinar cell proliferation.[4]

Besides Ptf1a, mature acinar cells express many other acinar specific transcription factors, including Mist1, which is required for exocrine granule maturation, Gata6, which is required for mature acinar cell homeostasis, and Nr5a2, that is crucial for the maintenance of acinar identity. The absence of the last two transcription factors results in the loss of acinar cell phenotype.[5]

Progenitor markers that drive ductal cell development have not been identified yet. Studies showed that a number of transcription factors like Sox9, Hnf1β, Hnf6 are involved in regulating specific aspects of ductal cell development during pancreas formation, however, none of them is specific for ductal cells only. Moreover, postnatal ductal cells express markers such as cytokeratin 19, cystic fibrosis transmembrane receptor, carbonic anhydrase II together with embryonic transcription factors. However, their expression is not uniform throughout different types of ducts, suggesting differences in ductal cells themselves. [6] More studies are necessary to elucidate the molecular mechanism of ductal cell formation and maturation.

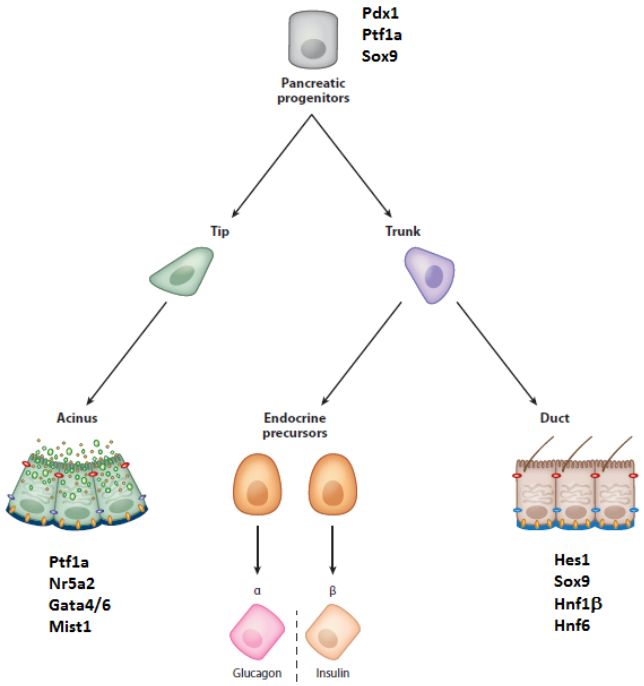


**Figure 2: Acinar and duct dells development during pancreas embryogenesis. (Picture is taken from [1])**

## 3.2 Pancreatitis

While diabetes is the most prevalent disease of the pancreas, pancreatic cancer, which arises in the exocrine tissue, is far more aggressive and lethal compared to diabetes. Moreover, pancreatitis, which is a disease of the exocrine tissue associated with high morbidity and mortality, is considered a risk factor to develop pancreatic cancer. Taking into account these aspects, it is fair to highlight the importance of studies investigating the mechanism of pancreatitis.

*Pathology of pancreatitis*

Pancreatitis is an inflammatory disease of the pancreas that manifests itself in both acute and chronic forms. Acute pancreatitis presents clinically with abdominal pain and nausea and is diagnosed by measuring elevated lipase and amylase levels in the circulation. There are two forms of acute pancreatitis: a mild one, which resolves within days or weeks, and a severe one, which can be lethal due to multiorgan failure. Acute pancreatitis can be caused by a duct obstruction with gallstones, alcohol or tobacco abuse, infection or trauma. Chronic pancreatitis is most commonly associated with alcoholism and presents itself with a persistent abdominal pain. Acute and chronic pancreatitis can be linked, as recurrent

inflammatory events progress to a chronic disease. The low level of circulating digestive enzymes in chronic pancreatitis is thought to be an indicator of acinar cell atrophy. [7]

The incidence of pancreatitis ranges from 13 to 45 for the acute form and from 5 to 12 for the chronic form per 100 000 people. [8, 9] Pancreatitis prognosis is variable, according to the severity of the disease and despite its relative rarity, pancreatitis continues to cause significant morbidity and mortality throughout the world.

*Mechanism of pancreatitis*

Following pancreatic injury early changes occur in acinar cells, including a block in secretion, co-localization of zymogens and lysosomal enzymes, intracellular activation of trypsinogen and other zymogens that result in acinar cell injury. As a result of injury, acinar cells release chemokines and cytokines, which initiate the recruitment of inflammatory cells into the tissue. In very severe cases, uncontrollable autodigestion results in the extensive tissue necrosis.

Multiple animal models of pancreatic injury exist, but the model of choice for studying the mechanisms of pancreatitis has become cerulein-induced pancreatitis. Cerulein is an analog of cholecystokinin (CCK), which is a major gastrointestinal hormone with a central role in pancreatic secretion. Physiological doses of CCK mediate pancreatic secretion and growth and do not cause pancreatitis. In contrast, supramaximal doses (i.e. concentrations exceeding the dose that induces a maximal amylase secretion) of CCK or cerulein inhibit pancreatic enzyme secretion and result in zymogen activation and acinar cell injury. A block in secretion alters pancreatic digestive enzyme and lysosomal hydrolase segregation and causes their co-localisation. This co-localisation results in trypsinogen activation by lysosomal cathepsin B, leading to autodigestion of the acinar cell. The importance of catepsin B during pancreatitis was shown by cathepsin B-deficient mice where trypsin activity was 80% lower than in wild-type mice. However, the pancreatic injury was decreased only by 50% in cathepsin B-deficient mice, suggesting that cathepsin B is not the only hydrolase involved in zymogen activation.[10]

*Inflammation*

Cerulein stimulated secretion leads to overproduction of digestive enzymes and induction of endoplasmic reticulum (ER) stress. ER stress leads to immune cell attraction, most likely via NFκB-dependent cytokine synthesis. [7] NFκB is a regulator of cytokine induction and, in turn, a modulator of inflammation. During pancreatitis various cytokines and chemokines are synthesized and released, for example TNF-$\alpha$, IL-6, IL-1$\beta$, MCP-1. Studies have demonstrated that pancreatic acini release significant amounts of TNF-$\alpha$ as early as 30min after cerulein stimulation and this leads to neutrophils attraction to the site of injury. [11] The significance of neutrophils in the pathogenesis of pancreatitis has been illustrated by reduced severity of pancreatitis following their depletion. Neutrophil penetration into the tissue is followed by invasion of macrophages, then T and B cells and in the last stage dendritic cells

[12]. The recruitment and activation of various inflammatory cells leads to further exacerbation of acinar cell injury caused by elevated synthesis of various pro-inflammatory mediators, such as IL-1, IL-2, TGF-β and ICAM-1. All these pro-inflammatory molecules also induce a fibrotic response by activating pancreatic stellate cells that are discussed in more detail in the next section.

*Pancreatic fibrosis*

As mentioned above, acute pancreatitis is a transient insult. However, when the injury is persistent or recurrent, the acute form of pancreatitis transforms to chronic. While acute pancreatitis is histologically described by oedema, acinar vacuolisation and fat or acinar cell necrosis, chronic pancreatitis is characterised not only by the mentioned features but also by persistent acinar cell atrophy, inflammation and fibrosis. Fibrosis is a typical feature of chronic pancreatitis (see Figure 3) defined by the accumulation of excessive amount of extracellular matrix (ECM) proteins. It develops primarily in the perilobular space where necrosis was located. Pancreatic stellate cells (PSCs) play an important role in fibrosis and, upon activation by pro-inflammatory mediators, produce collagen and other ECM proteins. In resting state, PSCs are located in the periacinar and interlobular area, contain lipid droplets and store vitamin A. [13] PSCs also express vimentin, desmin and nerve growth factor, but hardly any ECM proteins. TGF-β1, PDGF, FGF-2, TNF, IL-1 and IL-6 activate PSCs, which then acquire a myofibroblast-like phenotype, with the disappearance of their lipid droplets and the expression of α-smooth muscle actin. In parallel, stimulated PSCs proliferate, migrate and deposit ECM proteins and matrix metallo-proteinases. [14] PSCs are also involved in perpetuating chronic inflammation. Indeed, activated PSCs synthesize and secrete growth factors and cytokines that attract inflammatory cells and thus linking the inflammatory response with fibrosis.

*Treatment of pancreatitis*

As of today, no specific causal treatments for pancreatitis exist. The goals of treatment for pancreatitis are to relieve pain, calm the disease process to prevent recurrent attacks, correct metabolic consequences such as diabetes and malnutrition, and manage complications when they arise. The main therapeutic approaches to pancreatitis are restricted to regular analgesics, maintaining an adequate intravascular volume by rehydration, diet adjustments, surgery and sometimes antibiotic intake in order to manage infections. [9] The lack of an effective cure is partially linked to a limited understanding of the disease progression at the molecular level. In addition, the treatment of pancreatitis would also benefit from the availability of better diagnostic tools, because patients present themselves at a late stage of disease when the injury began to progress to a systemic phase.

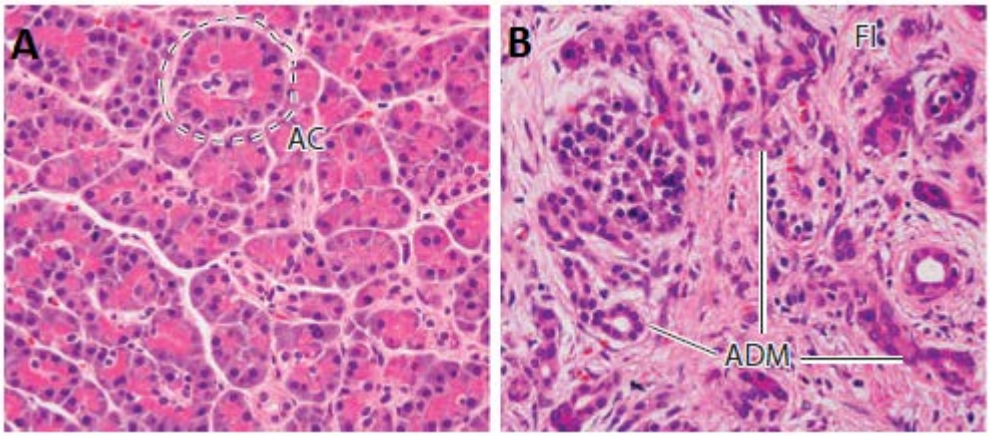


**Figure 3: The normal and injured pancreas. (a) A section of normal human pancreatic tissue with the outlined cluster of acinar cells (AC). (b) A section of human chronic pancreatitis specimen with indicated areas of fibrosis (FI) and acinar-to-ductal metaplasia (ADM). (Picture is taken from [6])**

## 3.3 Acinar-to-ductal metaplasia (ADM)

*Introduction to pancreatic metaplasia*

Pancreatitis is often associated with metaplasia, which is detected in the diseased pancreas in both humans and animal models. Metaplasia is generally defined as a transformation of one tissue type into another [15]. Cells can be transformed into other cell types by either dedifferentiation or transdifferentiation. Dedifferentiation is a reversion of terminally differentiated cell to a less-differentiated stage within its own linage. Transdifferentiation indicates that a cell regresses to a point where it can switch lineages, allowing it to differentiate into another cell type. [16] Injury induced to the pancreas leads to the formation of acinar-to-ductal metaplasia (ADM), which presents itself as ductal-like lesions in pancreatic tissue (see Figure 3). ADM is thought to arise from acinar cells that lose their phenotype and form ductal structures. Cells within ductal structures have not only morphological similarities with pancreatic ducts but these cells also express ductal markers and, more importantly, re-express pancreatic progenitor markers, which are active during pancreas development.

Morphologically, ADM is classified in [17]:

- Type 1 tubular complexes (TC1), which have a wide lumen lined by very few flat cells
- Type 2 tubular complexes (TC2), which are lined by many cells arranged in groups with branching.

*Acinar cell changes during ADM formation*

Pancreatic injuries and manipulations can induce a loss of the hallmark characteristics that identify a terminally differentiated acinar cell. During this process, acinar cells lose their specific proteins, like amylase, lipase and trypsin, and later shut down their expression totally. Also, a morphological remodelling of acinar cells takes place, which includes cytoplasm contraction and cytoskeleton rearrangement. Finally, a switch in gene expression

is induced where the developmental or repair program is activated with the expression of genes that are active during pancreatic embryogenesis and in uninjured pancreas are restricted to duct cells, for instance Hes1, Hnf6 and Sox9. Despite the fact that acinar cells dedifferentiate to structures similar to ducts, which express many ductal and progenitor markers, these structures are not considered as real ductal or progenitor cells because they lack the expression of other markers, including Hnf1β and Nkx6.1 [18]. Recent studies showed that the extent and degree of dedifferentiation depends upon the duration of injury, suggesting that this is a protective response reducing the harmful effects of digestive enzymes and simultaneously generating a cell with progenitor-like qualities. Notably, the dedifferentiation process depends on interactions between acinar cells and immune cells, which almost immediately from the time of injury participate in the process acting as critical regulators of ADM [7].

*Regulators of ADM*

Among the first events occurring during pancreatic injury is an influx of inflammatory leukocytes, which amplify the severity of the injury. Macrophages are one set of immune cells that are associated to acinar cells undergoing metaplasia and their depletion before pancreatitis significantly reduces ADM formation [19]. In contrast to macrophages, T and B cells did not show any effect regarding ADM formation, however, the resolution of ADM is impaired when the immune system lacks both T and B cells [20]. Moreover, pancreatic injury is associated with abundant production of inflammatory cytokines, which originate and act on both acini and leukocytes. Cytokine implication in ADM formation has been shown in transgenic mice overexpressing IL-1β, LTαβ or TGFα, where each of these molecules was sufficient to induce ADM [21-23]. TGFα is a ligand of the epidermal growth factor receptor (EGFR), the activation of which causes not only ADM formation both *in vitro* and *in vivo,* but also accelerates pancreatic tumorigenesis [24]. However, how EGFR exactly promotes ADM remains incompletely understood and requires further investigations.

Embryonic signalling pathways, active during pancreas organogenesis, are normally excluded from adult acinar cells but become reactivated upon injury. Analysis of animal models revealed that Notch, Hedgehog (HH), Hippo and Wnt/β-catenin signalling pathways are re-expressed in pancreas following injury. Notch and HH signalling pathways, which exert opposite effects during pancreatic development, have a similar role after pancreatic injury and promote ADM resolution and acinar cell re-differentiation [25, 26]. The Hippo pathway is necessary for maintaining acinar cell identity and, in the absence of Mst1 and Mst2 Hippo kinases, acinar cells dedifferentiate forming ADM. The Wnt/β-catenin pathway promotes acinar cell proliferation during pancreas formation and is necessary for ADM resolution [27]. All in all, the immunity and the developmental signalling pathways appear to

play opposite roles: the first is important in promoting ADM and the second one in resolving it.

As already mentioned above, there are many pancreatic transcription factors (TF), which are differentially activated and regulated in three states of the organ: pancreas development, adult pancreas and injured pancreas. Many of them have been studied to investigate how their absence or presence in combination with or without injury, influences ADM formation or resolution. For example, the potency of ductal TFs HNF6 and Sox9 to induce ADM was analysed by adenoviral overexpression [28]. While HNF6 overexpression was sufficient to induce ADM, Sox9 overexpression could only destabilise acinar cells by forcing them to express ductal cell marker cytokeratin 19. However, when conditional Sox9 deletion was coupled with cerulein pancreatitis it did not reveal any significant difference in ADM formation between knockout and control animals [29]. These results indicate different capacity of ductal TF to dedifferentiate acinar cells: HNF6 is a more potent inducer of metaplasia compared to Sox9, which alone cannot drive ADM formation.

Another set of TFs regulating ADM formation includes Mist1, GATA6 and Nr5a2, which are required for normal acinar cell differentiation. Inactivation of Mist1 by overexpression of dominant-negative form or by genetic deletion led to induction of ductal cell marker expression [30]. When the genetic deletion was coupled with cerulein pancreatitis, this accelerated metaplasia formation. Particularly critical TFs for maintaining acinar cell identity are GATA6 and Nr5a2, the disruption of which abolished acinar cell terminal differentiation [31, 32]. All these TFs bind to and activate most of acinar specific genes and together constitute a master regulatory network for acinar differentiation. A disruption of one of these TFs is sufficient to down-regulate acinar specific genes and activate the ductal phenotype.

*Importance of ADM*

The studies discussed above indicate the remarkable ability of acinar cells for plasticity and cell fate changes, meaning that these cells can transiently or stably adopt duct-like characteristics. This capability depends on molecular and environmental factors that reactivate developmental programs in response to injury or immune reaction.

Why are acinar cell plasticity and ADM important? The main reasons are pancreatitis and pancreatic cancer. Pancreatitis is associated with ADM and its resolution depends on re-differentiation and re-establishment of normal acinar cell function. Studies investigating pancreatic cancer indicate that precursor lesions of this disease arise from ADM [33]. Moreover, many of the same genes and pathways that promote metaplasia also promote the development of pancreatic cancer. Many studies describing the plasticity of acinar cells during pancreatic diseases highlight the fact that by understanding the mechanisms of metaplasia, new therapeutic approaches could be identified for pancreatitis and pancreatic cancer treatment.

## 3.4 Aims of the project

The exact molecular mechanisms regulating the development of ADM are not completely elucidated. Pancreatic injuries induce ADM formation but, what is more important, also force acinar cells and notably ADM itself to proliferate. To investigate and better characterize ADM we analysed how pro- or anti-proliferative factors affect ADM. Specifically, to investigate ADM formation we addressed the following questions:

1. What is the role of $p21^{Waf1/Cip1}$ in ADM formation? $p21^{Waf1/Cip1}$ is a powerful inhibitor of cell proliferation, as it controls cell cycle progression at G1-S and G2-M transitions. We hypothesised that in the absence of $p21^{Waf1/Cip1}$ proliferation should increase not only in acinar cells but in ADM areas as well, possibly leading to ADM expansion. To answer this question we used WT and $p21^{Waf1/Cip1}$ knockout mice, which were exposed to different regimens of cerulein treatment in order to induce pancreatitis.
2. What is the role of TGF-β signalling in acinar cell proliferation and ADM formation during pancreatitis progression? One of many TGF-β signalling functions is induction of cell cycle arrest through cyclin-dependent kinase (Cdk) inhibitors. We hypothesised that inhibition of TGF-β signalling in pancreatic epithelial cells should increase acinar cell proliferation and possibly ADM formation. To analyse the role of TGF-β signalling in pancreatic epithelial cells we used a conditional, pancreas specific TGF-β receptor II (TGF-β RII) knockout mouse model. To induce pancreatitis we exposed control and TGF-β RII knockout mice to different regimens of cerulein and analysed pancreatic regeneration.
3. What is the role of serotonin in ADM formation? Serotonin is a potent mitogen with a pro-proliferative effect in many adult tissues. We hypothesised that in the absence of serotonin proliferation of pancreatic acinar cells and concomitant ADM formation should be diminished. To investigate serotonin-dependent effects in pancreatic tissue we used transgenic mice deficient in peripheral serotonin ($TPH1^{-/-}$). We compared these mice with WT animals in two regeneration models, which differed in the magnitude of regenerative stimuli: in one model, tissue injury was induced by cerulein treatment and in the other, tissue loss was obtained by partial pancreatectomy.

# 4. Manuscript A

## p21$^{WAF1/Cip1}$ limits senescence and acinar-to-ductal metaplasia formation during pancreatitis

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**Contribution:**

This study represents a major part of my PhD work. All experimental work was done by me (except confocal microscopy analysis performed by Adrian B. Hehl) and a minority of experiments were done in collaboration with colleagues. I also contributed to drafting/revising of large parts of the manuscript.

# $p21^{WAF1/Cip1}$ limits senescence and acinar-to-ductal metaplasia formation during pancreatitis

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**Running title:** p21 and ADM

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## Abstract

Trans-differentiation of pancreatic acinar cells into ductal-like lesions, a process defined as acinar-to-ductal metaplasia (ADM) is observed in the course of organ regeneration following pancreatitis. In addition, ADM is found in association with pre-malignant PanIN lesions and correlates with an increased risk of pancreatic adenocarcinoma (PDAC). Human PDAC samples show down-regulation of $p21^{WAF1/Cip1}$, a key regulator of cell cycle and cell differentiation. Here we investigated whether p21 down-regulation is implicated in controlling the early events of acinar cell trans-differentiation and ADM formation. p21-mediated regulation of ADM formation and regression was analyzed *in vivo* during the course of cerulein-induced pancreatitis using wild type (WT) and p21 deficient ($p21^{-/-}$) mice. Biochemical and immunohistochemical methods were used to evaluate disease progression over two weeks of the disease and during a recovery phase. We found that p21 was strongly up-regulated in WT acinar cells during pancreatitis, while it was absent in ADM areas, suggesting that p21 down-regulation is associated with ADM formation. In support of this hypothesis, $p21^{-/-}$ mice showed a significant increase in number and size of metaplasia. In addition, p21 over-expression in acinar cells reduced ADM formation *in vitro,* suggesting that the protein regulates the metaplastic transition in a cell-autonomous manner. $p21^{-/-}$ mice displayed increased expression and re-localization of β-catenin during both pancreatitis and subsequent recovery phase. Finally, loss of p21 was accompanied by increased DNA damage and development of senescence. Our findings are consistent with a gate-keeper role of p21 in acinar cells to limit senescence activation and ADM formation during pancreatic regeneration.

**Keywords:** senescence; p21; ADM; β-catenin; pancreatitis

## Introduction

Pancreatitis is a severe inflammatory disease of the pancreas and its chronic and hereditary forms are a major risk factor for developing pancreatic ductal adenocarcinoma, a human malignancy associated with a 5-year survival rate of less than 5% [1]. A key step in the malignant transformation is the formation of acinar-to-ductal metaplasia (ADM), where acinar cells de-differentiate into a progenitor state characterized by the appearance of tubular complexes and reactivation of transcription factors and signaling pathways typical of pancreatic development [2-4]. Indeed, recent lineage tracing studies confirmed that reprogramming of differentiated acinar cells to ADM contributes to the formation of pre-malignant lesions [5]. Importantly, a transient ADM formation and concomitant acinar cell proliferation has also been observed during pancreatitis both in humans and rodents, suggesting that the plasticity of acinar cells and their ability to de-differentiate into facultative

progenitor cells are crucial processes for pancreatic regeneration.

The molecular mechanisms regulating the development of metaplasia are not completely elucidated and their characterization would advance our knowledge not only of pancreatic regeneration but also of the progression to malignant lesions.

Here, we investigated molecular factors which limit ADM formation. Specifically, we focused on $p21^{WAF1/Cip1}$, a molecule with a complex network of intracellular functions. The molecule is known to inhibit cyclin/CDK activity, regulate apoptosis and senescence, and control transcription of specific genes [6-8]. Importantly, p21 is down-regulated in human pancreatic ductal adenocarcinoma [9, 10]. In addition, certain p21 polymorphisms are associated with increased risk of pancreatic adenocarcinoma [11], and improved survival was observed for patients whose adenocarcinoma was p21 positive [12], suggesting that p21 may indeed influence the susceptibility to and the outcome of pancreatic cancer.

To address the role of p21 in ADM formation, we evaluated acinar trans-differentiation *in vitro* upon p21 over-expression in acinar cells and *in vivo* upon genetic ablation of p21. In the latter case, we used the controlled setting of cerulein-induced pancreatitis, which allows the analysis of metaplasia formation without the added complexity of cancer progression present in murine models of pancreatic cancer.

## Materials and methods

### *Animal experiments*

All animal experiments were performed in accordance with Swiss Federal animal regulations and approved by the cantonal veterinary office of Zurich. Pancreatitis was induced in adult (8-10 weeks of age) wild-type B6;129SF1/J and $p21^{-/-}$ (B6;129S2-Cdkn1a<tm1Tyj>/J, The Jackson Laboratories, USA) mice via six intra peritoneal (i.p.) injections of 50 μg/kg cerulein administered hourly over a two week period, as described in Supplementary Materials and Methods and [13]. Control animals received 0.9% NaCl injections.

### *Histology, immunohistochemistry and immunoblotting*

Detailed protocols and primary antibodies used in this study are listed in Supplementary Materials and Methods. Quantification of labelled cells was performed in at least ten randomly selected high-power fields (×100) per slide. Non-acinar tissue areas (islets, vessels, fibrotic tissue) were excluded from analysis.

### *Transcript analysis*

Total RNA was extracted from pancreata as previously described [14] and reverse transcribed with qScript™ cDNA SuperMix (Quanta Biosciences). Transcript levels were normalized using 18S rRNA as a reference and expressed as fold induction relative to the value of untreated control animals. Taqman probes (Applied Biosystems) used in this study are listed in Supplementary Materials and Methods.

### *Adenoviral infections*

Cultures of primary acinar explants isolated according to [15] and rat pancreatic AR42J cells (ATCC, CRL-1492) were maintained as described in Supplementary Materials and Methods. Adenovirus expressing p21 (Adp21) (rat) or green fluorescent protein (AdGFP) were purchased from Vector Biolabs (Philadelphia, PA) and added to acinar explants before embedding in collagen at the multiplicity of infection (MOI) described in the figure legends.

### *Confocal microscopy and image analysis*

Analysis of fluorescently labeled β-catenin subcellular distributions was performed on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) using a glycerol immersion objective lens (Leica, HCX PL APO CS 63x 1.3 Corr). Single optical sections in the central z-plane of the sample were collected with a pinhole setting of Airy 2 using constant settings for laser output and photomultiplier sensitivity. Both were adjusted to collect all signals within the linear range. Quantification of signal output within each micrograph (histogram quantification) was performed using the Leica applications software suite. The absolute signal output and the mean intensity in four arbitrarily positioned squares (regions of interest, ROI) of ~8'500 $\mu m^2$ within each of the 9 recorded micrographs per condition were measured in three biological replicates. To quantify the distribution of markers within the tissue section, i.e. plasma membrane/nuclei-associated vs. cytoplasmic signal (profile quantification), six arbitrarily positioned profile scans of ~70 μm length in each of the nine separate micrographs per condition were made. For statistical analysis the mean signal amplitudes and the mean variances of six profiles were calculated for each micrograph.

## Results

### p21 is up-regulated in acinar cells but not ADM during pancreatitis

To investigate the function of p21 in ADM formation, we first assessed its expression in human samples of pancreatic diseases. p21 was abundantly expressed in areas with still intact acinar cells during chronic pancreatitis (Fig.1A a). However, in human pancreatic ductal adenocarcinoma, p21 expression was comparatively modest in peri-

tumoral lesions (Fig.1A b, c) and in advanced tumors [9, 10], suggesting that down-regulation of p21 is either a consequence of acinar trans-differentiation and malignant transformation or it a prerequisite for the cells to transform.

To determine whether down-regulation of p21 is required for acinar trans-differentiation, we analysed the temporal and spatial regulation of p21 in mice following cerulein-induced injury, as this murine model allows us to investigate the initiating events of ADM formation without the added complexity of tumor progression. While p21 was barely detectable in the uninjured pancreas, its acinar expression (Fig. 1B) and transcript levels (Fig. 1C) increased robustly during cerulein treatment. Non-acinar compartments, including islets and ducts and interstitial cells, remained negative for p21 throughout the treatment (Fig. 1B, central panel, S1A). A negative control for the staining with the secondary antibody alone is shown in Fig. S1A. Moreover, transcript levels of p21 transcriptional activators, including p53, TGFβ1 and its receptor TGFβ-RII, increased following cerulein treatment (Fig. 1C). Mist1, which directly induces p21 expression in pancreatic acinar cells [16], also increased one week after cerulein treatment (Fig. 1C), following an initial transient reduction (Fig. S1B). Amylase, a down-stream target of Mist1, exhibited similar expression dynamics (Fig. S1B). These observations suggest that p21 expression may be induced via multiple pathways during pancreatitis. In stark contrast with the high expression of p21 observed in acinar cells, only minor amounts of the protein were detected in ADM areas, characterized by loss of acinar organization and tubular complex formation (Fig. 1B, asterisks).

**ADM is enhanced in the absence of p21**

As we observed low expression of p21 in ADM, we hypothesize that its down-regulation is a pre-requisite for metaplastic transition. To test this hypothesis, we used mice lacking p21 ($p21^{-/-}$) and quantified ADM lesions during cerulein-induced pancreatitis. Lack of p21 is compatible with normal development and pancreatic functionality [17], and results only in a modestly increased rate of spontaneous tumor development at advanced age [18-20]. Untreated $p21^{-/-}$ mice showed pancreata with normal cytoarchitecture and differentiated acinar cells with amylase content comparable with WT mice (Fig. 4B, C), indicating that p21 is not required for mouse pancreatic development and physiology. Eight hours after pancreatitis induction, serum levels of amylase (Fig. S2A) and lipase (Fig. S2B) were higher in $p21^{-/-}$ than in WT mice. Previous work showed that deletion of the p21 inducer Mist1 resulted in higher enzymemia and up-regulation of CCK receptor A [21, 22]. However, CCK-RA expression levels did not increase in the absence of p21 (Fig. S2C). WT and $p21^{-/-}$ mice showed comparable tissue morphology, as seen by H&E staining (Fig. S2D). In addition, eight hours after the beginning of the treatment both strains had similar up-regulation of apoptosis, quantified by TUNEL assay and cleaved caspase 3 (Fig. S3A, B), and autophagy, quantified by LC3-II western blotting and p62 immunostaining [23] (Fig.S3C, D). Infiltration of inflammatory cells (Fig. S3E, F) and cytokine/chemokine expression (Fig. S10A) was also comparable in the two strains. However, after 4 days and one week of cerulein treatment, $p21^{-/-}$ mice presented more frequent and extended ADM lesions than WT animals, which then resolved after two weeks of treatment (Fig. 2A, B). As expected, immunohistochemical analyses confirmed that p21 was absent in $p21^{-/-}$ mice (Fig. S4A). The prominent metaplastic lesions in $p21^{-/-}$ mice showed typical ADM features, including down-regulation of amylase and its transcription factor p48, cytokeratin accumulation and phospho-Stat3 expression. In addition, they were surrounded by collagen-rich stroma and activated stellate cells expressing α-smooth muscle actin (αSMA) (Fig. 2C, D, S4B), as previously shown [24]. They expressed Sox9 and CD44, a type I transmembrane cell surface protein target of the APC-β-catenin-TCF pathway [25], and used as a marker for putative pancreatic cancer stem cells [26, 27]. Overall transcript levels of progenitor markers (Fig. S4C), as well as Sox9 expression in intact acinar cells (Fig. S4D), were also slightly enhanced in the absence of p21. To investigate whether p21 directly regulates ADM formation, we overexpressed p21 by adenovirus infection in WT primary acinar explants embedded in three-dimensional fibrillar collagen and induced trans-differentiation by stimulation with TGF-α [28]. P21 over-expression reduced ADM formation *in vitro* (Fig. 2E), indicating that increased levels of p21 in acinar cells are sufficient to limit acinar trans-differentiation.

**Acinar cell proliferation does not increase in the absence of p21**

p21 is a powerful inhibitor of cell proliferation, as it controls cell cycle entry by binding to and inhibiting cyclin-dependent kinases operating in both G1/S and G2/M transition check points [8, 29]. Thus we evaluated whether the increased ADM formation in the absence of p21 is the result of increased acinar cell replication. Expression of both early (D1, E1) and late (A1, B1) cyclins, were significantly higher in $p21^{-/-}$ mice compared to WT animals after cerulein treatment (Fig. 3A, B). Similarly, transcript levels of both Cdc25B (Fig. S5A), and Cdc25C (Fig. 3C, left panel), phosphatases that regulate the activity of cyclin B-Cdc2 kinase complex and cell cycle progression at the $G_2$/M boundary [30], were higher in $p21^{-/-}$ mice, while expression of Cdc25A, which regulates the $G_1$/S transition, was comparable in the two strains (Fig. S5A). Furthermore, activated Cdc25C, measured by the level of its phosphorylated form [31], was higher in

the absence of p21 (Fig. 3C, right panel). We found that p21 was expressed in proliferating acinar cells positive for the cell cycle activation marker Ki67 (Fig. S5B, C). However, lack of p21 did not increase acinar cell proliferation, as seen by enumeration of cells positive for Ki67 (Fig. 3D, S6A), G1/S phase marker PCNA (Fig. S6A) and mitosis marker phospho-histone H3 (pH3) (Fig. S6A, B). Importantly, we did not observe any difference in proliferation between intact acinar cells and ADM (Fig. 3E), indicating that the two different types of epithelial cells have comparable replication rates, which are not regulated by p21. These data indicate that, in response to injury, key cell cycle activators are up-regulated in mice lacking p21, yet the rate of mitosis is similar in both genotypes. Thus, activation of compensatory mechanisms may limit acinar cell proliferation in $p21^{-/-}$ mice.

**Lack of p21 does not compromise acinar recovery following pancreatitis**

Next we analysed whether the ability of the pancreas to recover from tissue injury is altered in the absence of p21. After a week of recovery following one week cerulein treatment, pancreata from both WT and $p21^{-/-}$ mice showed a normal histological phenotype indistinguishable from untreated animals (Fig. 4A, S7A). Similarly, the transition from the temporarily de-differentiated acinar cells to the terminally differentiated phenotype was efficient in the absence of p21, as shown by the restored amylase content quantified by immunostaining (Fig. 4B), immunoblotting (Fig. 4C) and enzymatic assay in both tissue and serum (Fig. S7B, C). ADM lesions recovered as well, and expression of their markers was reduced, as shown by Sox9 and CD44 staining (Fig. 4D, E) and autophagy was not detected (Fig. S7D). Collectively, these data show that lack of p21 allows not only a prompt de-differentiation during injury but also does not compromise the ability of the tissue to overcome the cerulein-induced damage.

**Lack of p21 results in increased β-catenin expression and re-localization**

To further characterize the molecular mechanisms responsible for the p21-dependent regulation of ADM formation, we investigated β-catenin, a transcriptional activator of the canonical Wnt pathway implicated in the regulation of differentiation and regeneration of acinar cells [2, 32, 33]. Of note, β-catenin activation is sufficient to induce ADM formation [34]. As previously reported, quantitative confocal microscopy showed that cerulein treatment of WT mice up-regulated β-catenin expression, which further increased during the week of recovery (Fig. 5A, B). β-catenin was mostly cytosolic in untreated mice, while it re-localized to the plasma membrane and nuclei of acinar cells after pancreatitis induction (Fig. 5C), suggesting activation of Wnt signal transduction. Furthermore, β-catenin was present both in intact acini and ADM, where it presumably regulates CD44 expression [25] (Fig. 5D), indicating that β-catenin-dependent signalling is not limited to metaplastic areas but influences the whole tissue. We observed that $p21^{-/-}$ mice displayed stronger β-catenin labeling than WT mice after one week cerulein treatment and its expression, quantified by signal intensity, remained stable during the following recovery week (Fig. 5A, B). In addition, we quantified the localization of β-catenin in plasma membranes and nuclei versus cytosol in the two strains, by measuring the variance of signal amplitude, as exemplified in Fig. 5E, left panels. β-catenin re-localization to either plasma membranes or nuclei significantly increased in $p21^{-/-}$ mice compared with WT animals after one week cerulein treatment and became more cytosolic during the recovery time (Fig. 5E, right panel), suggesting faster dynamics of intracellular re-localization of the protein in the absence of p21. To investigate whether p21 directly regulates the expression of β-catenin, we overexpressed p21 by adenovirus infection in AR42J acinar cells. These cells express β-catenin, mainly localized to the plasma membrane and nuclei, while p21 was present at low levels (Fig. S8A). p21 overexpression did not change the levels of β–catenin (Fig. S8B), or the viability of AR42J cells (Fig. S8C), suggesting that p21 does not directly regulate β–catenin expression in this cell type. In addition, β-catenin expression and Wnt signaling were previously found to be inhibited or activated by p53 [35-38]. qPCR analyses showed similar p53 expression in WT and $p21^{-/-}$ mice (Fig. S8D), suggesting that β-catenin up-regulation is not driven by aberrant p53 regulation.

**Lack of p21 results in increased DNA damage and senescence in acinar cells**

Expression and activation of β-catenin signaling has recently being shown to increase following DNA damage [39]. In addition, oxidative stress and DNA damage are regarded as major pathogenic factors in human and cerulein-induced pancreatitis [40]. Thus, we investigated whether loss of p21 was associated with increased DNA damage that could contribute to the observed β-catenin up-regulation. Expression of γH2AX, an early hallmark of active DNA damage response [41], was detected in mouse and human samples of chronic pancreatitis (Fig. 6A), while absent in untreated mice. γH2AX foci were more numerous in acinar cells and ADM areas of $p21^{-/-}$ mice after one week of pancreatitis (Fig. 6A, S9A). As DNA damage is a common cause of senescence [42], we tested whether senescence was promoted in absence of p21. Activity of acidic senescence-associated *β*-galactosidase (SA-*β*-gal), a canonical marker of senescence (recently reviewed in [42, 43]) was negligible in untreated $p21^{-/-}$ mice (Fig. 6B), indicating that lack of p21 does not activate a

senescence program. However, following cerulein treatment these mice showed higher enzyme activity compared with WT animals, indicating that lack of p21 increased injury-induced senescence. Furthermore, additional senescence biomarkers, including the cyclin-dependent kinase inhibitors p16, were up-regulated in intact acini of $p21^{-/-}$ mice one week after experimental pancreatitis (Fig. 6C, S9B). Expression of the cyclin-dependent kinase inhibitor p27 increased in the absence of p21 (Fig. S9C), while the levels of cyclin B/Cdk1 inhibitors Myt1 and Wee1 were comparable in the two strains (Fig. S9D), indicating that expression of these factors is not altered in the absence of p21. In parallel with the increased DNA damage, the number of apoptotic cells both in intact tissue and ADM areas was more pronounced in the absence of p21 at one week of cerulein treatment (Fig. 6D). Finally, as senescent cells develop a secretory phenotype (SASP), which includes pro-inflammatory factors such as IL-1β and IL-6 [44], we tested whether the increased senescence observed in the absence of p21 was accompanied by increased tissue inflammation. As shown in Fig. S10A, $p21^{-/-}$ mice did not show increased levels of pro-inflammatory transcripts one day after cerulein injection. However, concomitantly with the development of senescence after one week of treatment, $p21^{-/-}$ mice had increased cytokine/chemokine expression and recruitment of inflammatory cells in the pancreas (Fig. S10A, B), without increased fibrotic processes and activation of stellate cells (Fig. S11A-C). Altogether, these results show increased senescence in the absence of p21, suggesting that up-regulation of this protein during pancreatitis protects acinar cells from excessive accumulation of DNA damage and subsequent entry into a senescent state or cell death.

**Discussion**

p21 was initially characterized as a cyclin-dependent kinase (CDK) inhibitor, however, further studies revealed that this molecule participates in a broader range of biological functions, spanning from regulation of DNA synthesis to stress response and control of cell differentiation [8]. Here we show that p21 was up-regulated in acinar cells during the development of pancreatitis and that its lack resulted in higher incidence of DNA damage and cellular senescence. Senescence is often mediated via independent activation of either p53/p21 or p16/pRb, but these pathways can cooperate [45] or substitute each other [46]. Previous studies associated p21 and p16 expression with senescence in acinar cells [47]. However, our data showed that p21 protects acinar cells from senescence and its loss results in increased senescence and expression of p16, thus confirming previous findings of p16 as a key regulator of senescence in the pancreas [48]. Collectively, our data suggest that p21 up-regulation during pancreatitis induces a temporary quiescence that may allow DNA repair and protects acinar cells against entry into a senescence program. Of note, a similar role of p21 in establishing quiescence and limiting senescence has been shown in few cell types, including human fibroblasts [49], murine skeletal muscle cells and adipocytes [50]. Conversely, p16 was shown to protect from DNA damage during p21-induced senescence in cells of the hematopoietic system, the small intestine, and the testes [51]. These recent reports highlight the observation that the senescence program can be differently regulated in different organ systems.

In this study we also demonstrated an inverse correlation between the amount of p21 and the level of metaplastic transition, as lack or up-regulation of p21 resulted in increased or decreased ADM formation, respectively. Thus p21 may act as a tumor-suppressor in the adult pancreas. However, ADMs were transient also in $p21^{-/-}$ mice and acinar cells were able to re-activate the differentiation program, indicating that the absence of p21 alone is not sufficient to maintain a de-differentiated phenotype. The increased incidence of ADM formation observed in the absence of p21 may result from different mechanisms. First, lack of p21 may increase the susceptibility of acinar cells to damage. $p21^{-/-}$ mice had increased enzymemia eight hours after pancreatitis induction. However, tissue histology, apoptosis, autophagy and inflammation were comparable in the two strains in the acute phase of the disease, suggesting that lack of p21 is not associated with elevated cellular damage. Second, absence of p21 could affect the functionality of non-acinar cells, including stromal cells, whose activity may be necessary for proper regeneration of the injured parenchyma. Indeed, pancreatic stellate cells showed increased p21 expression *in vitro* during senescence, a process which increases the efficiency of immune-mediated cytolysis and terminates pancreatic fibrogenesis [52]. Although we cannot dismiss an interaction with stromal cells completely, we found that expression of p21 in acinar cells is sufficient to regulate metaplastic transition in a cell-autonomous manner.

In addition, previous studies have associated increased ADM formation with a defect in acinar cell replication, as recently shown for the loss of the polycomb group proteins Bmi-1 and Enhancer of Zeste Homolog 2 (EZH2) or Notch1 inhibition [53-55]. Our results did not reveal a reduced mitotic rate in absence of p21, however the observed up-regulation of cyclins without increased expression of mitotic markers suggests that an aborted replication in acinar cells may contribute to the enhanced metaplasia. These observations also indicate that adult acinar cells have a tightly controlled replication and, as observed in several

transgenic cancer models, that these cells are resistant to transformation leading to a malignant phenotype [56]. Of note, pancreatic β cells showed similar resilience to exit G1/0 arrest even under replication stimuli and loss of p21 was not sufficient to overcome the inhibitory control of the cell cycle [17, 57]. This argues for the presence of redundant or compensatory mechanisms regulating cellular replication of pancreatic cells.

Finally, increased ADM may result from increased senescence developed in the absence of p21. The concept of senescence-induced ADM in the context of pancreatitis has not been thoroughly investigated yet. One intriguing possibility is that senescent cells secrete pro-inflammatory cytokines and proteases that may promote a microenvironment suitable for the development of ADM. In this context, we recently demonstrated that focal inflammation in the pancreas, independent from cerulein-induced injury, is sufficient to promote the formation of individual metaplastic lesions [58]. In addition, it is worth noting that several factors known to modulate ADM formation are also involved in senescence regulation. Specifically, Bmi-1 and EZH2, polycomb group transcriptional repressors that limit ADM in the pancreas [53, 55], are also known to inhibit senescence in different cells types [59, 60]. In addition, β-catenin, whose activation is sufficient to induce ADM formation [34], has recently been shown to mediate senescence and senescence associated secretory phenotype (SASP) in mesenchymal stem cells and cancer cells [61-63]. In our work, we found that absence of p21 is associated with an increase in β-catenin levels in the injured pancreas. Thus it is possible that following p21 loss, elevated β-catenin contributes to the increased senescence observed in the pancreas, and promotes the establishment of ADM during the course of pancreatitis.

In conclusion, we showed that, in the course of pancreatitis, p21 acts as a molecular switch to promote quiescence of acinar cells and to limit DNA damage, activation of senescence and ADM formation. These data provide a new perspective not only on the biological response to injury in the pancreas but also on the complex molecular events governing pancreatic regeneration and the development of metaplasia.

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**Contributors** The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript. KG, ES, GMS, KS, RAZ performing experiments, generation and analysis of data; ABH, confocal microscopy, generation and analysis of data; AD, MB and TR, animal generation and execution of experiments; RG and SS, study design; SS, writing of the manuscript; RG, KG, RAZ, ABH, drafting/revising the manuscript for content, analysis and interpretation of data. All authors approved the submitted version.

**List of abbreviations***:* ADM, acinar-to-ductal metaplasia; SASP, senescence associated secretory phenotype.

**List of online supporting material:**

Supplementary material and methods.

Figure S1. P21 staining and transcript analysis of Mist1 and amylase during cerulein treatment.

Figure S2. Serum enzyme levels, CCK receptor expression, tissue histology of p21 in WT and $p21^{-/-}$ mice after 7 days of cerulein treatment.

Figure S3. Apoptosis, autophagy, inflammation analyses during cerulein treatment.

Figure S4. Progenitor-cell markers regulation in the absence of p21.

Figure S5. qPCR of Cdc25B and Cdc25A and co-localization analyses during cerulein treatment.

Figure S6. Immunostaining and quantification of proliferation markers during cerulein treatment.

Figure S7. Analyses of acinar cell functionality after the recovery phase.

Figure S8. P21 overexpression in AR42J cells.

Figure S9. Cell cycle regulator expression in the absence of p21.

Figure S10. Quantification of inflammation in the absence of p21.

Figure S11. Quantification of fibrosis in the absence of p21.

**Figure legends**

**Figure 1**. **A**. Immunostaining of p21 showed that the protein is expressed in human samples of chronic pancreatitis (a), while it is only minimally present (arrows) in peri-tumoral areas of human pancreatic ductal adenocarcinoma (b, c). Inset in a, enlarged view of p21 staining in acinar nuclei. Hematoxylin-Eosin (H&E) staining is shown in a', b', c'. **B**. Pancreatitis was induced in wild type B6;129SF1/J (WT) mice with six injections of 50 μg/kg cerulein administered hourly. Animals received injections on Monday, Wednesday and Friday and were harvested on Tuesday after one set of injections (day 1), on Thursday after two sets of injections (day 3) or on Monday after three (day 7) or six (day 14) sets of injections, without cerulein

treatment on the same day. Images show up-regulation of p21 in acinar cells at day 7 after the beginning of cerulein treatment. Note the absence of staining in islets (red borders, i), ductal compartments (arrow), and interstitial cells (arrowheads). ADM areas (black borders, asterisk) in 7 day treated mice showed minimal p21 staining. Arrows indicate tubular complexes. **C**. qPCR of p21, p53, TGFβ, TGFβ receptor II and Mist1 levels in the pancreas of WT mice following 7 and 14 days of cerulein-induced pancreatitis. Results are average ± SEM (n=5). Scale bars: 50 μM.

**Figure 2**. ADM is enhanced in the absence of p21. **A**. Hematoxylin-Eosin (H&E)-stained sections of pancreata at the indicated time of cerulein treatment showed extended regions of ADM (asterisks) in $p21^{-/-}$ mice after 4 and 7 days of pancreatitis. **B**. Quantification of ADM number following cerulein-induced pancreatitis. Data are normalized by the pancreatic area. Numbers above the bars indicate the average size of ADMs expressed as percentage of the total area of the pancreas. Results are average ± SEM (n>5), *p<0.05. **C**. Immunohistochemical analyses of $p21^{-/-}$ mice after 7 days of pancreatitis revealed that ADM areas (asterisks) were negative for the acinar cell markers amylase and p48, were positive for the ductal marker pan-keratin and phospho-Stat3 (p-Stat3), contained collagen deposits and expressed the stem marker CD44. **D**. Co-localization analyses showed that ADMs expressed the progenitor marker Sox9 and were surrounded by stromal reaction with activated stellate (αSMA) and inflammatory (pan-leucocytes) cells. **E**. Primary acinar cells from WT mice were infected with adenovirus encoding either p21 (Adp21) or GFP (AdGFP) as control and embedded in collagen. TGFα-induced trans-differentiation was quantified after 7 days in culture. Results are average ± SEM (n=4), *p<0.05. Scale bars: 50 μM.

**Figure 3**. Acinar cell proliferation does not increase in the absence of p21. **A**. qPCR revealed up-regulation of early and late cyclins in $p21^{-/-}$ mice after cerulein treatment. **B**. Cyclin B immunostaining showed up-regulation of the protein in $p21^{-/-}$ mice after 7 days of cerulein treatment. **C**. qPCR of Cdc25C (right panel) and immunostaining of phospho-Cdc25C (left panel) showed up-regulation and activation of the protein in $p21^{-/-}$ mice after 7 days of cerulein treatment. **D**. Quantification of Ki67 positive pancreatic acinar (Ac) and interstitial (Int) cells showed comparable proliferation rates in WT and $p21^{-/-}$ mice. **E**. Quantification of Ki67 positive cells in intact acini and ADMs showed comparable proliferation rates in WT and $p21^{-/-}$ mice. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

**Figure 4**. Lack of p21 does not compromise acinar cell recovery. **A** H&E staining of pancreata showed that ADMs in $p21^{-/-}$ mice resolved to WT levels following one week of recovery. Immunostaining of pancreatic tissue (**B**) and immunoblotting (**C**) of 20 μg proteins showed up-regulation of amylase in $p21^{-/-}$ mice following one week of recovery. Nuclei are stained with DAPI (blue). Immunostaining with Sox9 (**D**) and CD44 (**E**) revealed an almost complete disappearance of progenitor markers following one week of recovery. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

**Figure 5**. Lack of p21 is associated with increased β-catenin expression and re-localization in acinar cells. **A**. Confocal analysis of β-catenin expression following 7 days of cerulein treatment (7 d) and recovery (7 d+rec). **B**. Quantification of β-catenin expression by measuring signal intensity as described in the Material and Methods section. **C**. Co-localization of β-catenin and DAPI indicating nuclear re-localization of the protein after 7 days of cerulein treatment. **D**. Co-localization of β-catenin (red) and CD44 (green) indicating β-catenin expression in ADM (asterisk). **E**. Quantification of intracellular β-catenin re-localization by measuring signal amplitude as described in the Material and Methods section. Left panel, examples of quantification in control and 7 day treated pancreata. Right panel, quantification of β-catenin re-localization (signal amplitude) during cerulein treatment and recovery. Note the increased signal amplitude in the treated samples indicating plasma membrane/nuclear re-localization of β-catenin. Results are average ± SEM (n=3), *p<0.05. Scale bars: 50 μM.

**Figure 6**. DNA damage and senescence increased in the absence of p21. **A**. γH2AX immunostaining (upper panel) and quantification of positive cells (bottom panel) revealed increased DNA damage response following pancreatitis in the absence of p21. Asterisk indicates ADM. Right upper panel shows γH2AX immunostaining in human samples of chronic pancreatitis **B**. SA-β-galactosidase staining (upper panel) and enzymatic quantification (bottom panel) showed increased enzyme activity in $p21^{-/-}$ treated animals. **C**. qPCR (upper panels) and immunostaining (lower panels) showed increased expression of CDK inhibitors of the INK family in $p21^{-/-}$ mice after cerulein treatment. **D**. TUNEL staining showed increased number of apoptotic cells in intact acini in $p21^{-/-}$ mice after 7 days of cerulein treatment (left panel). High level of apoptotic cells was also detected in ADM areas (asterisk, right panel). Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

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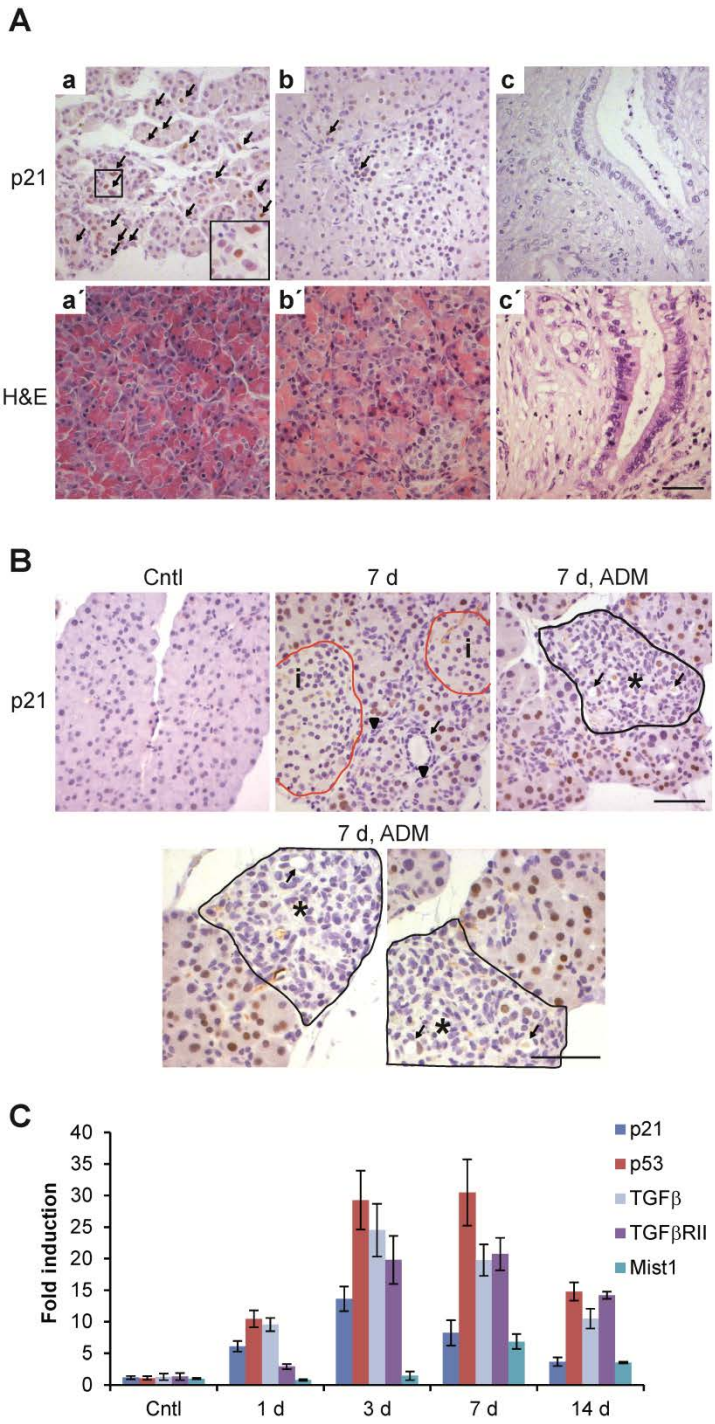

A
a
b
c
p21
a´
b´
c´
H&E
B
Cntl
7 d
7 d, ADM
p21
i
i
*
7 d, ADM
*
*
C
Fold induction
40
35
30
25
20
15
10
5
0
Cntl
1 d
3 d
7 d
14 d
p21
p53
TGFβ
TGFβRII
Mist1

**Fig. 1**

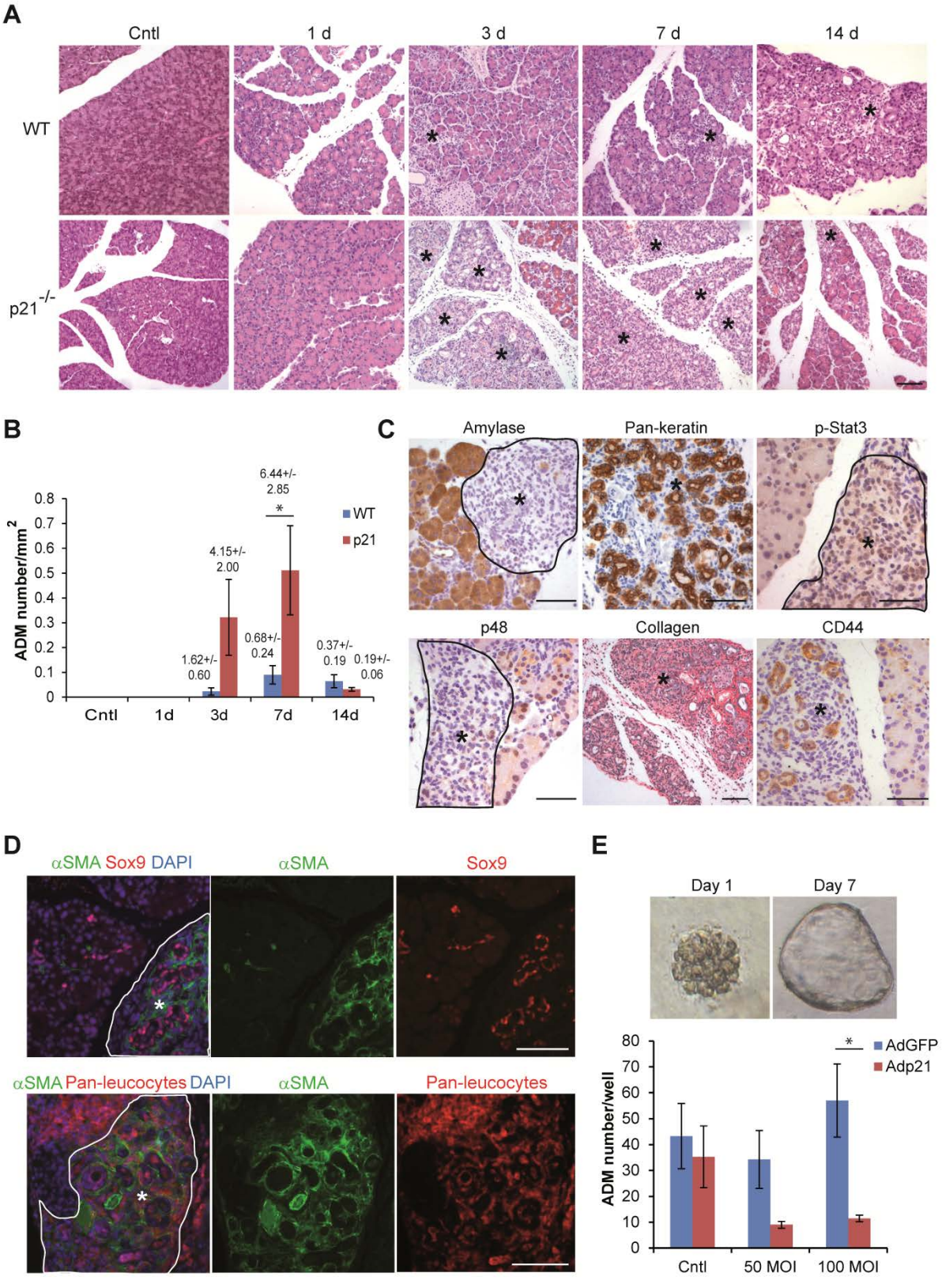

A
Cntl
1 d
3 d
7 d
14 d
WT
p21 -/-
B
ADM number/mm 2
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
6.44+/- 2.85
*
WT
p21
4.15+/- 2.00
1.62+/- 0.60
0.68+/- 0.24
0.37+/- 0.19
0.19+/- 0.06
Cntl
1d
3d
7d
14d
C
Amylase
Pan-keratin
p-Stat3
p48
Collagen
CD44
D
αSMA Sox9 DAPI
αSMA
Sox9
αSMA Pan-leucocytes DAPI
αSMA
Pan-leucocytes
E
Day 1
Day 7
ADM number/well
80
70
60
50
40
30
20
10
0
*
AdGFP
Adp21
Cntl
50 MOI
100 MOI

**Fig. 2**

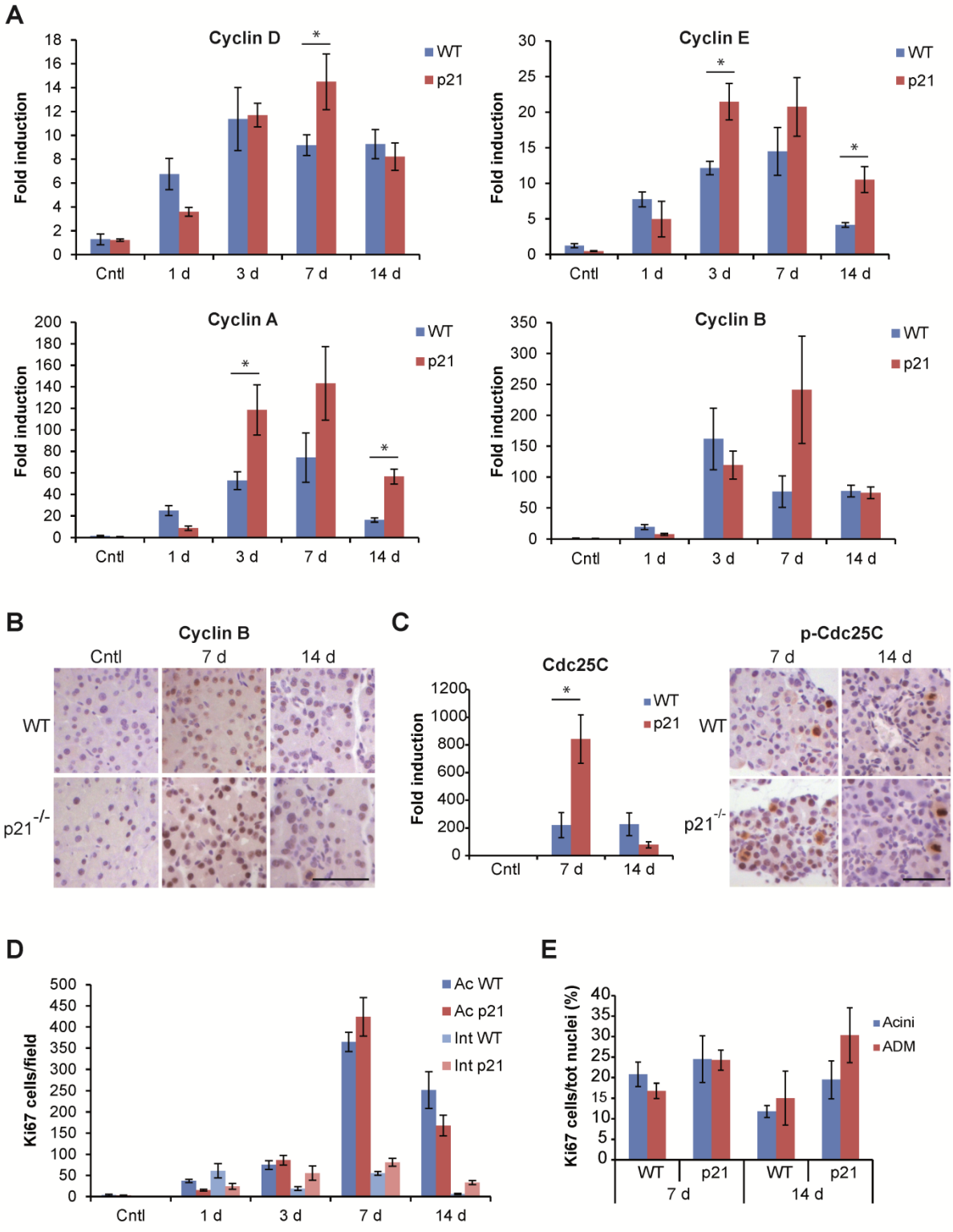

A
Cyclin D
Cyclin E
Cyclin A
Cyclin B
Fold induction
WT
p21
Cntl
1 d
3 d
7 d
14 d
B
Cyclin B
WT
p21-/-
C
Cdc25C
p-Cdc25C
D
Ki67 cells/field
Ac WT
Ac p21
Int WT
Int p21
E
Ki67 cells/tot nuclei (%)
Acini
ADM

**Fig. 3**

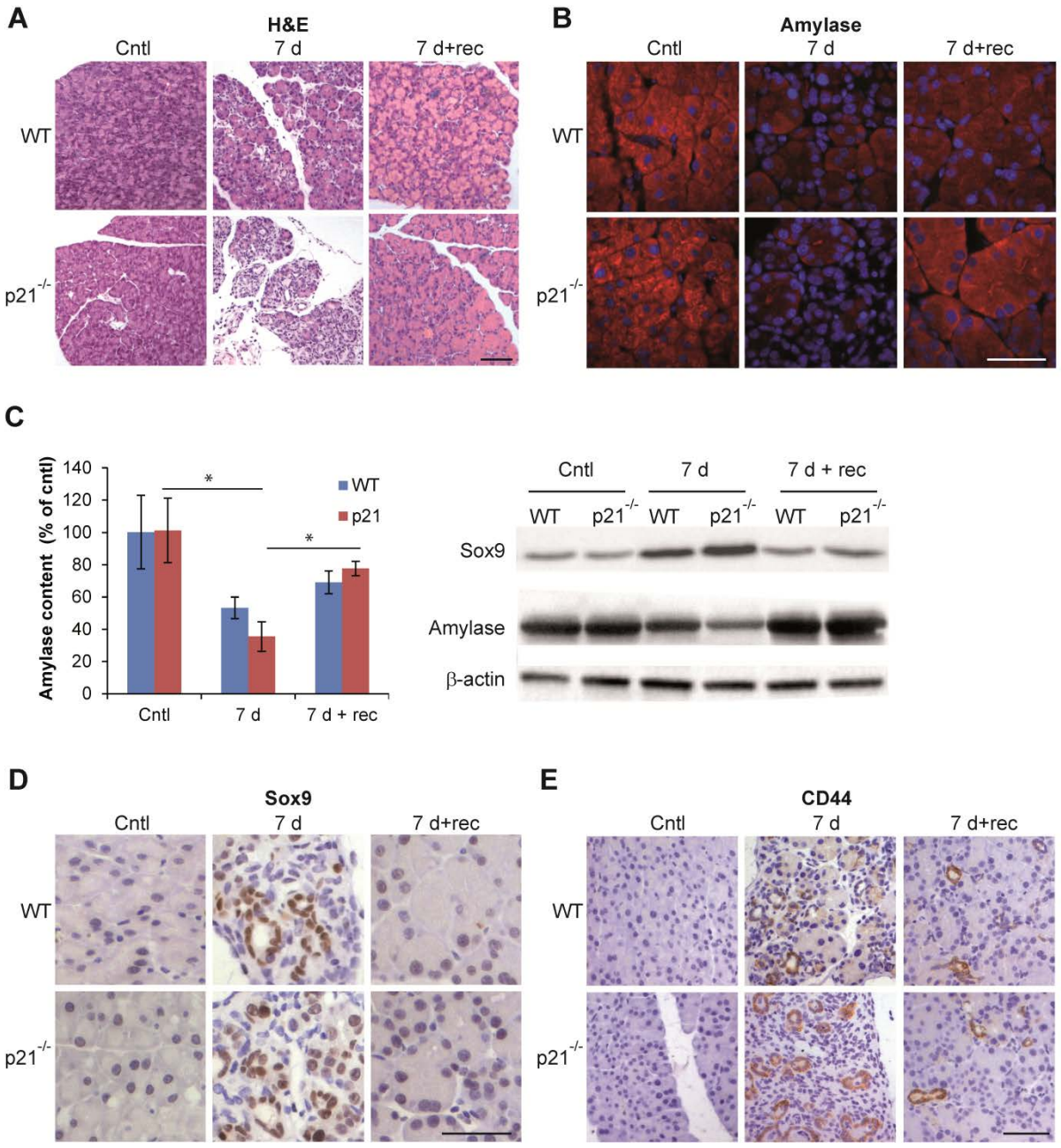

A
H&E
Cntl
7 d
7 d+rec
WT
p21-/-
B
Amylase
Cntl
7 d
7 d+rec
WT
p21-/-
C
Amylase content (% of cntl)
140
120
100
80
60
40
20
0
*
*
WT
p21
Cntl
7 d
7 d + rec
Cntl
7 d
7 d + rec
WT
p21-/-
WT
p21-/-
WT
p21-/-
Sox9
Amylase
β-actin
D
Sox9
Cntl
7 d
7 d+rec
WT
p21-/-
E
CD44
Cntl
7 d
7 d+rec
WT
p21-/-

**Fig. 4**

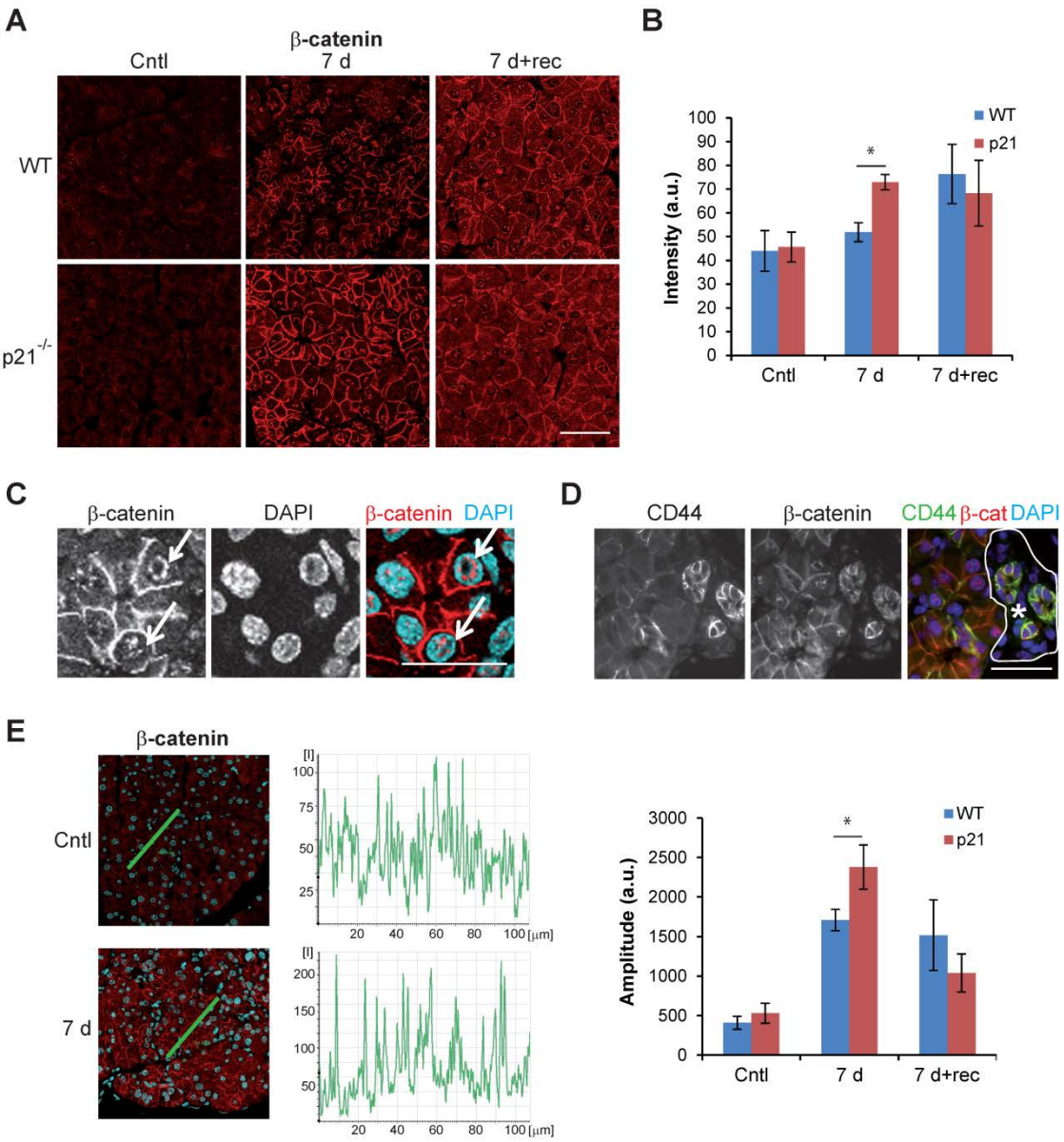

A
β-catenin
Cntl
7 d
7 d+rec
WT
p21-/-
B
Intensity (a.u.)
WT
p21
Cntl
7 d
7 d+rec
C
β-catenin
DAPI
β-catenin DAPI
D
CD44
β-catenin
CD44 β-cat DAPI
E
β-catenin
Cntl
7 d
[I]
[µm]
Amplitude (a.u.)
WT
p21
Cntl
7 d
7 d+rec

**Fig. 5**

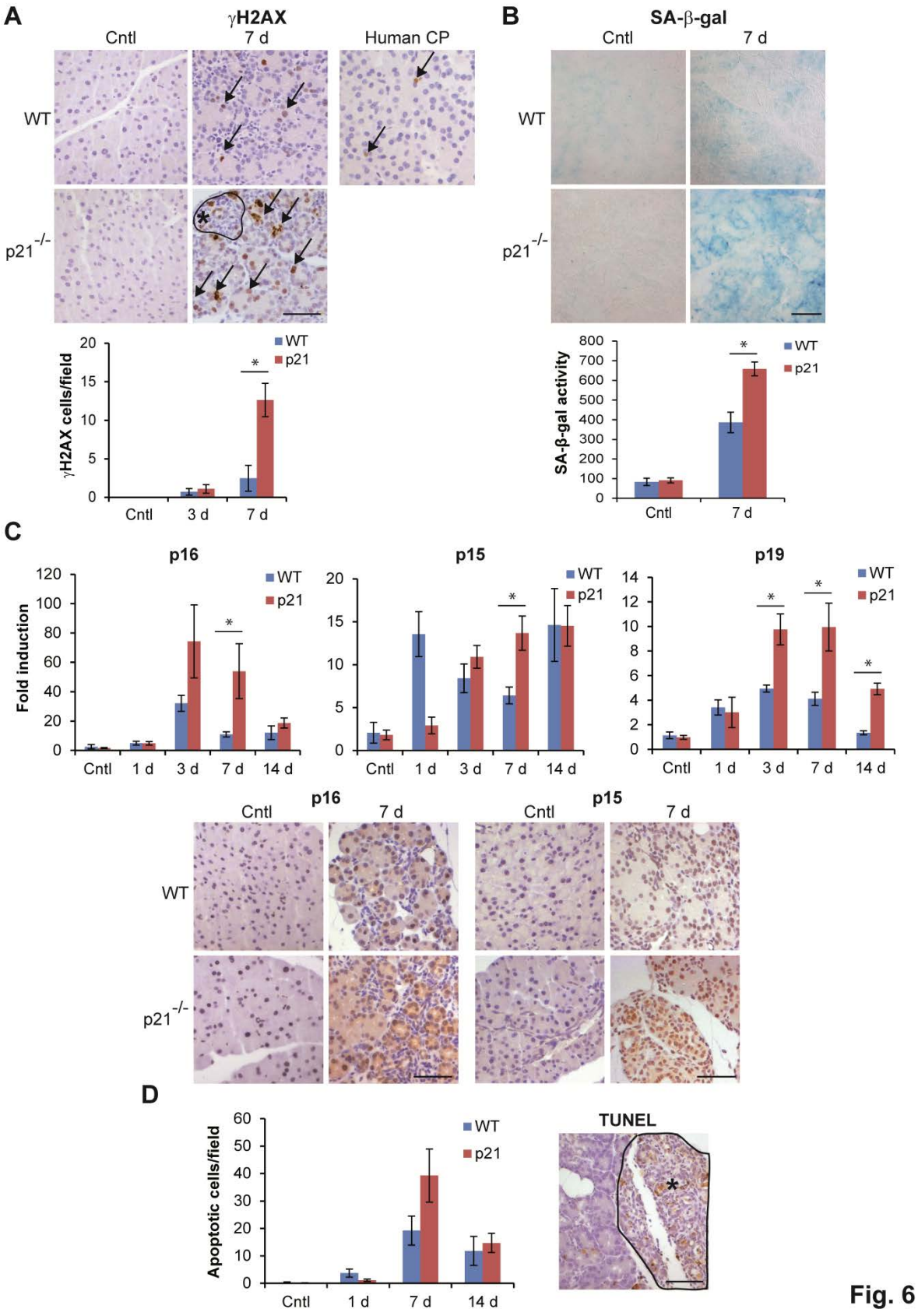

A
γH2AX
Cntl
7 d
Human CP
WT
p21-/-
γH2AX cells/field
WT
p21
Cntl
3 d
7 d
B
SA-β-gal
Cntl
7 d
WT
p21-/-
SA-β-gal activity
C
p16
p15
p19
Fold induction
Cntl
1 d
3 d
7 d
14 d
D
Apoptotic cells/field
TUNEL

Fig. 6

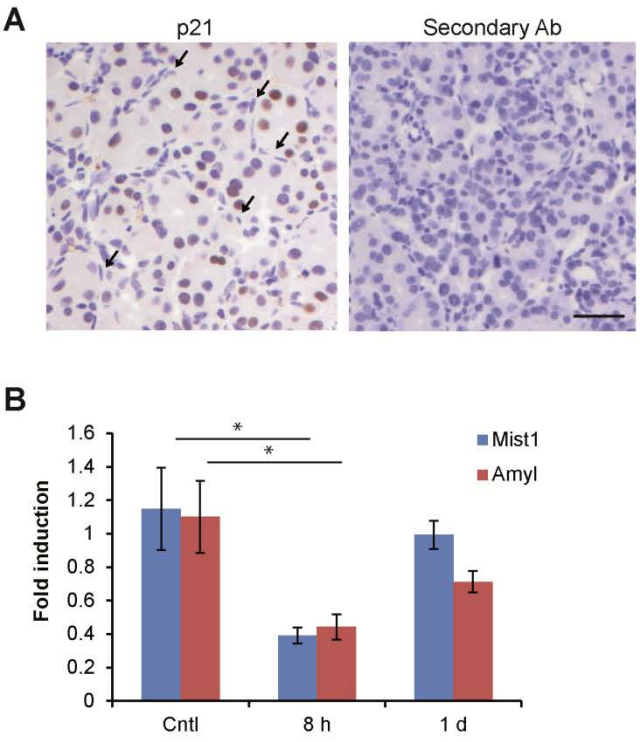

A
p21
Secondary Ab
B
Fold induction
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Mist1
Amyl
Cntl
8 h
1 d

**Fig. S1**

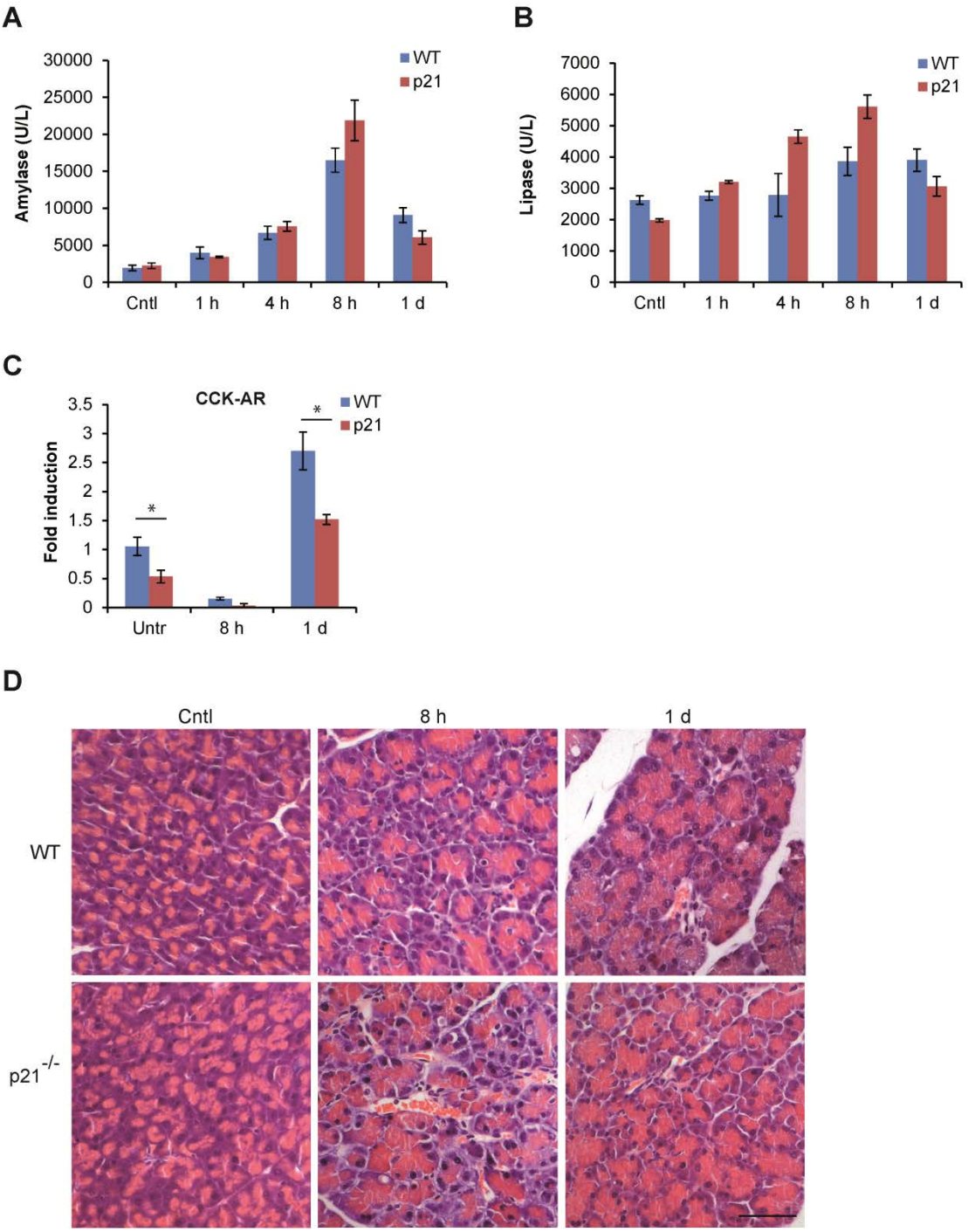

A
Amylase (U/L)
30000
25000
20000
15000
10000
5000
0
Cntl
1 h
4 h
8 h
1 d
WT
p21
B
Lipase (U/L)
7000
6000
5000
4000
3000
2000
1000
0
Cntl
1 h
4 h
8 h
1 d
WT
p21
C
CCK-AR
Fold induction
3.5
3
2.5
2
1.5
1
0.5
0
Untr
8 h
1 d
*
*
WT
p21
D
Cntl
8 h
1 d
WT
p21-/-

**Fig. S2**

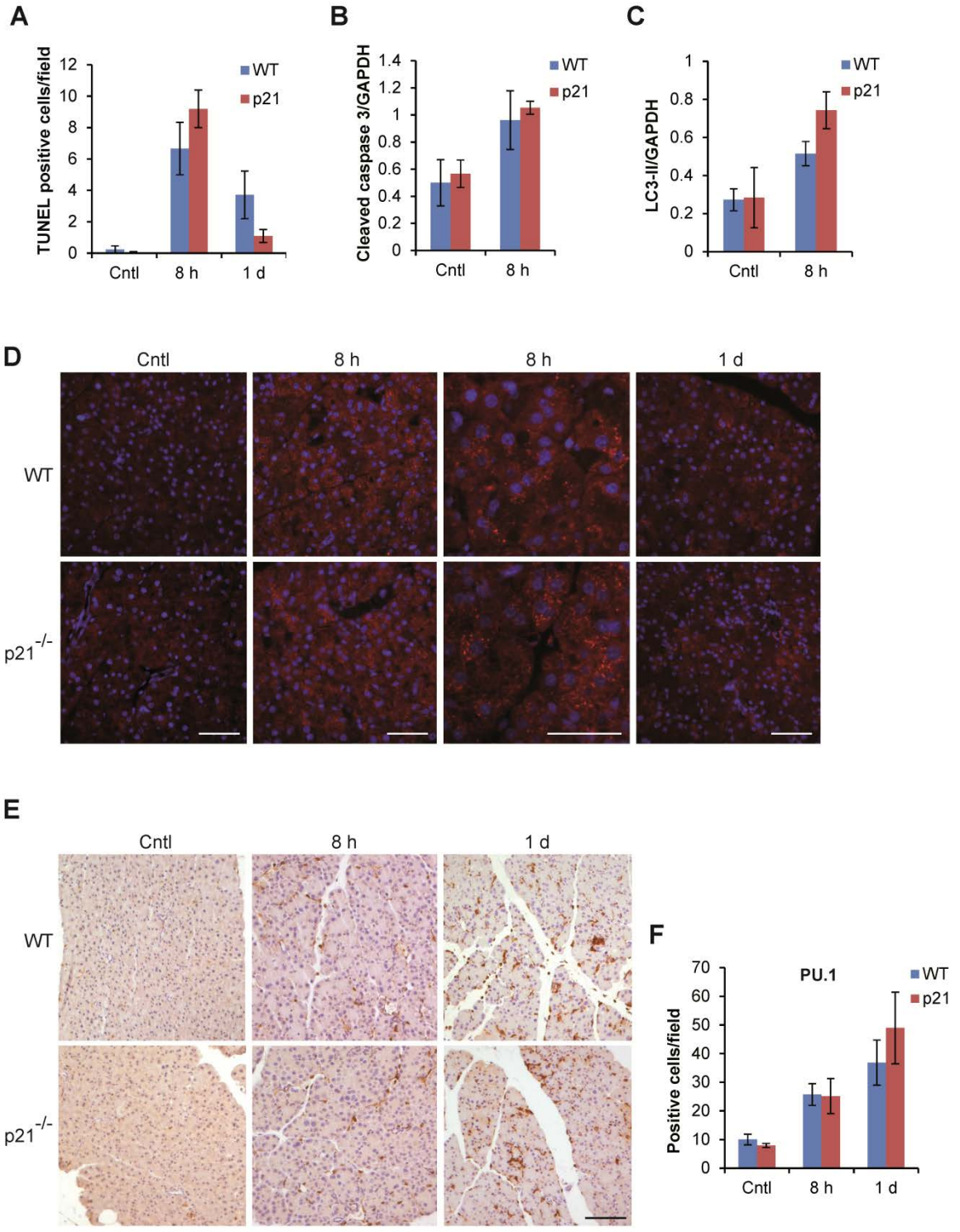

A
TUNEL positive cells/field
12
10
8
6
4
2
0
WT
p21
Cntl
8 h
1 d
B
Cleaved caspase 3/GAPDH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
p21
Cntl
8 h
C
LC3-II/GAPDH
1
0.8
0.6
0.4
0.2
0
WT
p21
Cntl
8 h
D
Cntl
8 h
8 h
1 d
WT
p21-/-
E
Cntl
8 h
1 d
WT
p21-/-
F
PU.1
Positive cells/field
70
60
50
40
30
20
10
0
WT
p21
Cntl
8 h
1 d

**Fig. S3**

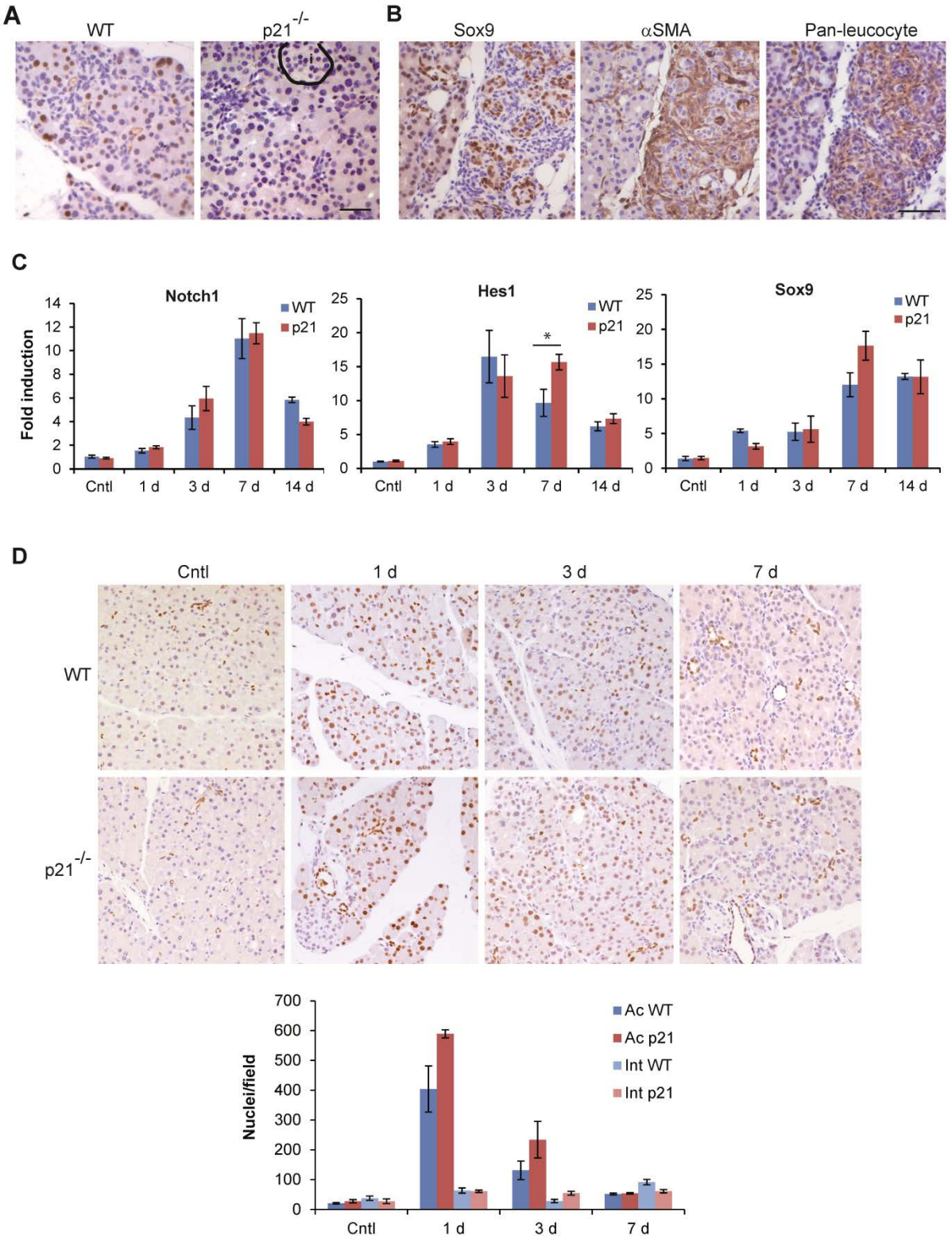

A
WT
p21-/-
B
Sox9
αSMA
Pan-leucocyte
C
Notch1
Hes1
Sox9
WT
p21
Fold induction
Cntl
1 d
3 d
7 d
14 d
*
D
Cntl
1 d
3 d
7 d
WT
p21-/-
Ac WT
Ac p21
Int WT
Int p21
Nuclei/field

**Fig. S4**

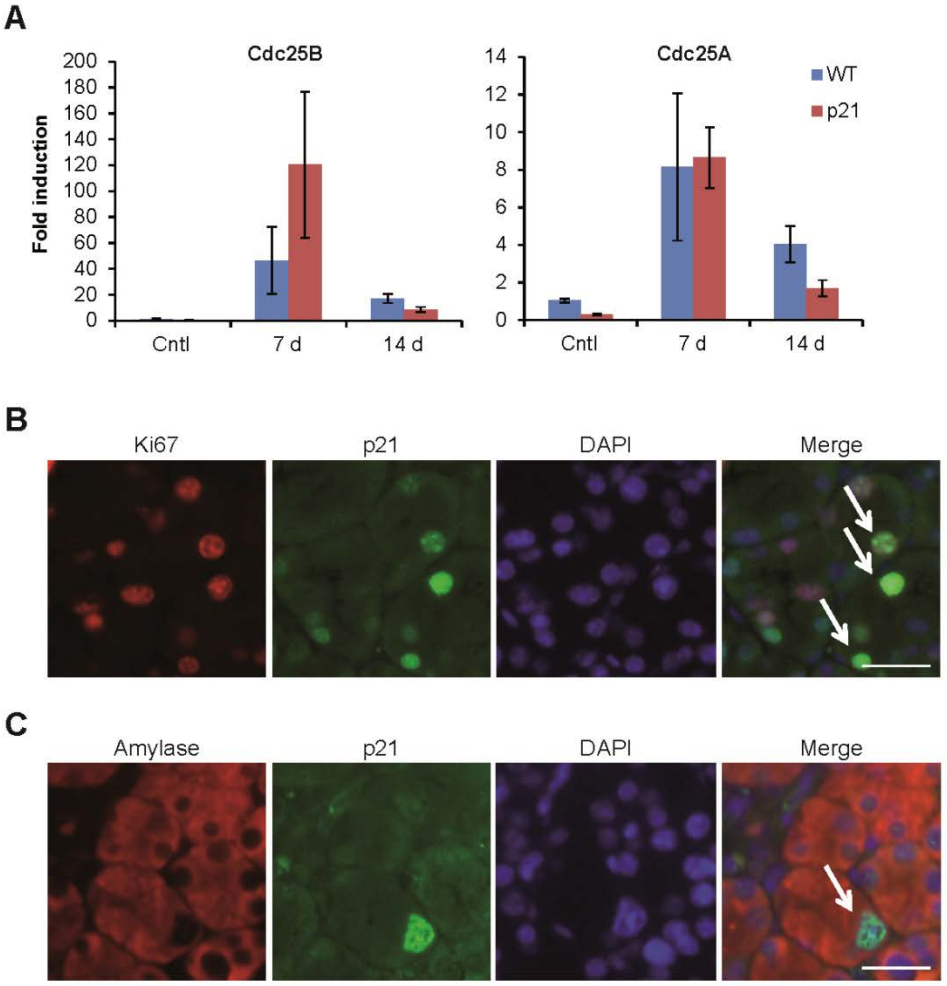
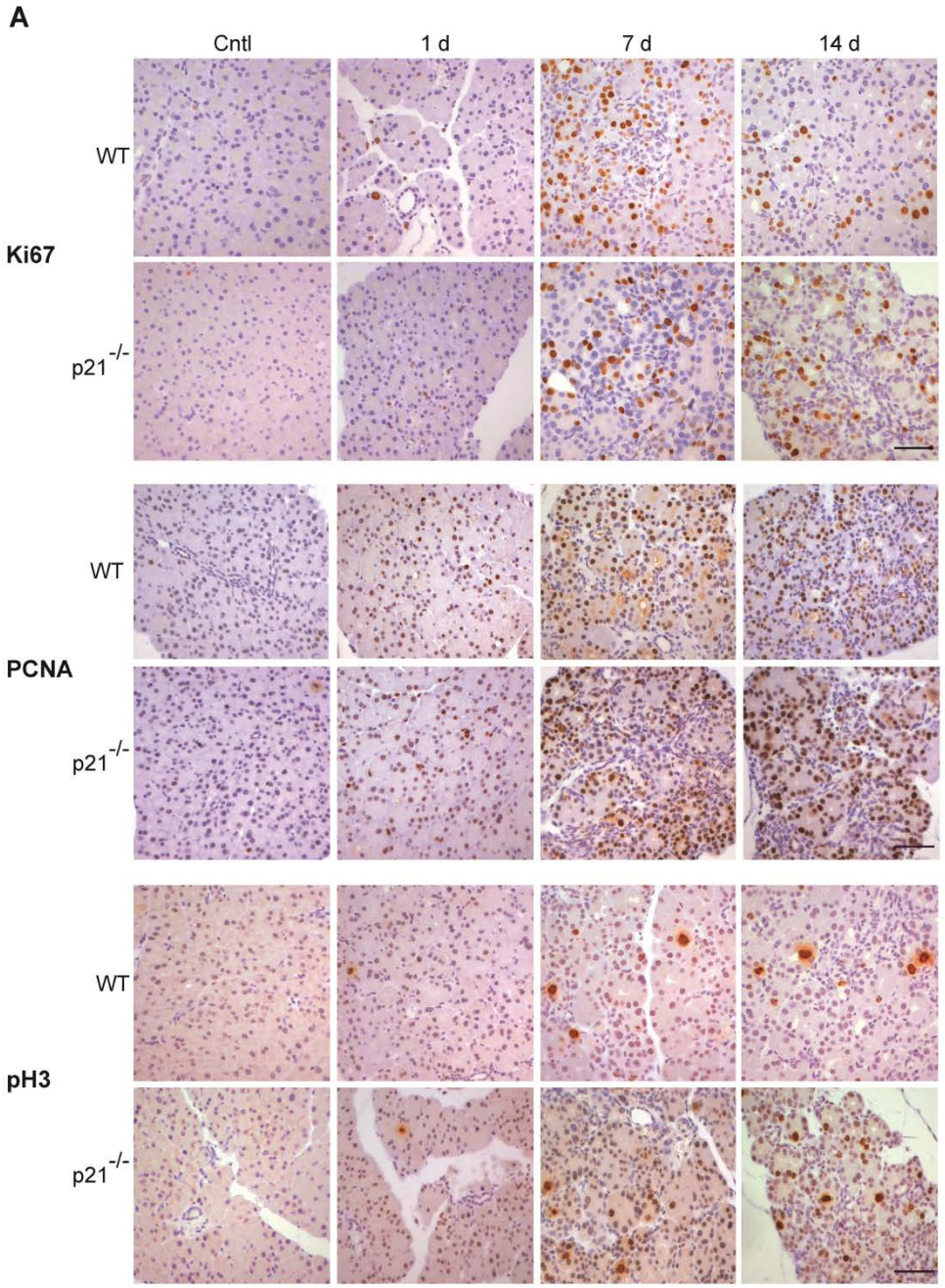

A
Cdc25B
Cdc25A
Fold induction
WT
p21
Cntl
7 d
14 d
B
Ki67
p21
DAPI
Merge
C
Amylase
p21
DAPI
Merge

**Fig. S5**


A
Cntl
1 d
7 d
14 d
WT
Ki67
p21-/-
WT
PCNA
p21-/-
WT
pH3
p21-/-

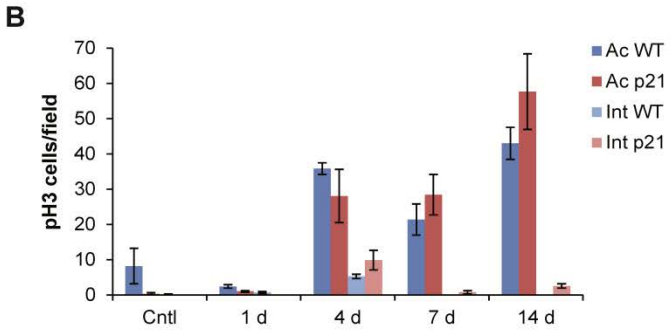

B
pH3 cells/field
70
60
50
40
30
20
10
0
Cntl
1 d
4 d
7 d
14 d
Ac WT
Ac p21
Int WT
Int p21

**Fig. S6**

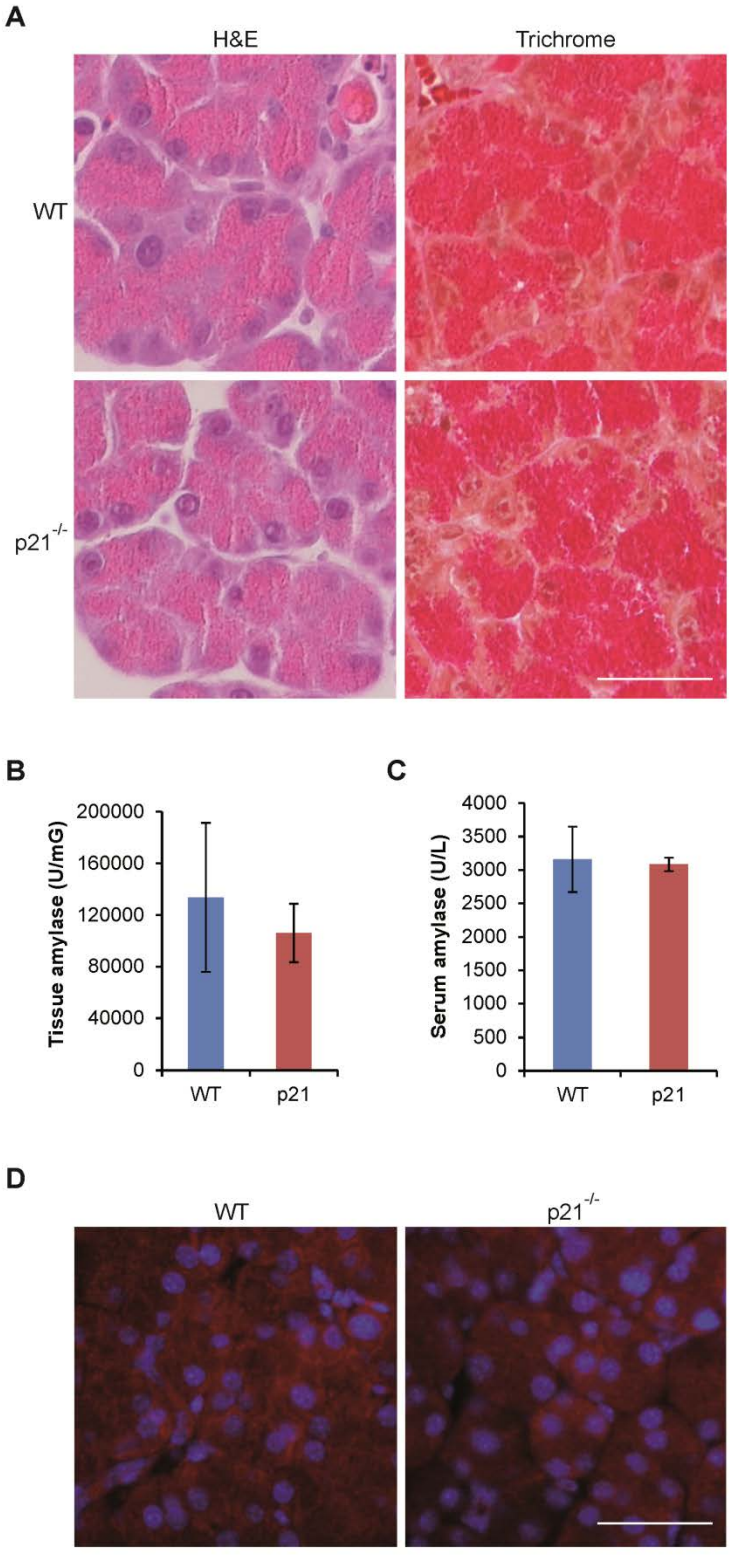

A
H&E
Trichrome
WT
p21-/-
B
Tissue amylase (U/mG)
200000
160000
120000
80000
40000
0
WT
p21
C
Serum amylase (U/L)
4000
3500
3000
2500
2000
1500
1000
500
0
WT
p21
D
WT
p21-/-

**Fig. S7**

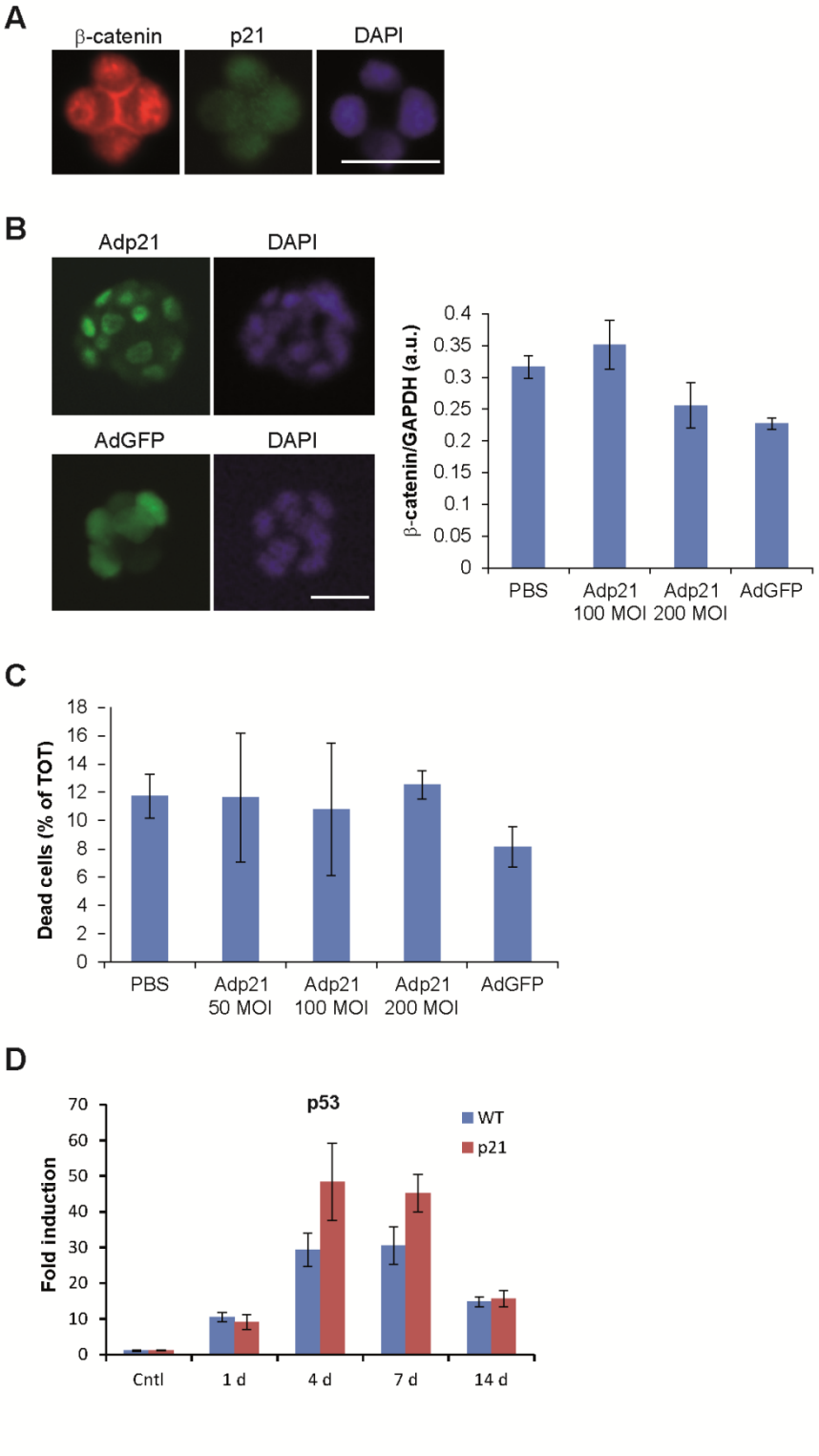

A
β-catenin
p21
DAPI
B
Adp21
DAPI
AdGFP
DAPI
β-catenin/GAPDH (a.u.)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
PBS
Adp21 100 MOI
Adp21 200 MOI
AdGFP
C
Dead cells (% of TOT)
18
16
14
12
10
8
6
4
2
0
PBS
Adp21 50 MOI
Adp21 100 MOI
Adp21 200 MOI
AdGFP
D
p53
WT
p21
Fold induction
70
60
50
40
30
20
10
0
Cntl
1 d
4 d
7 d
14 d

**Fig. S8**

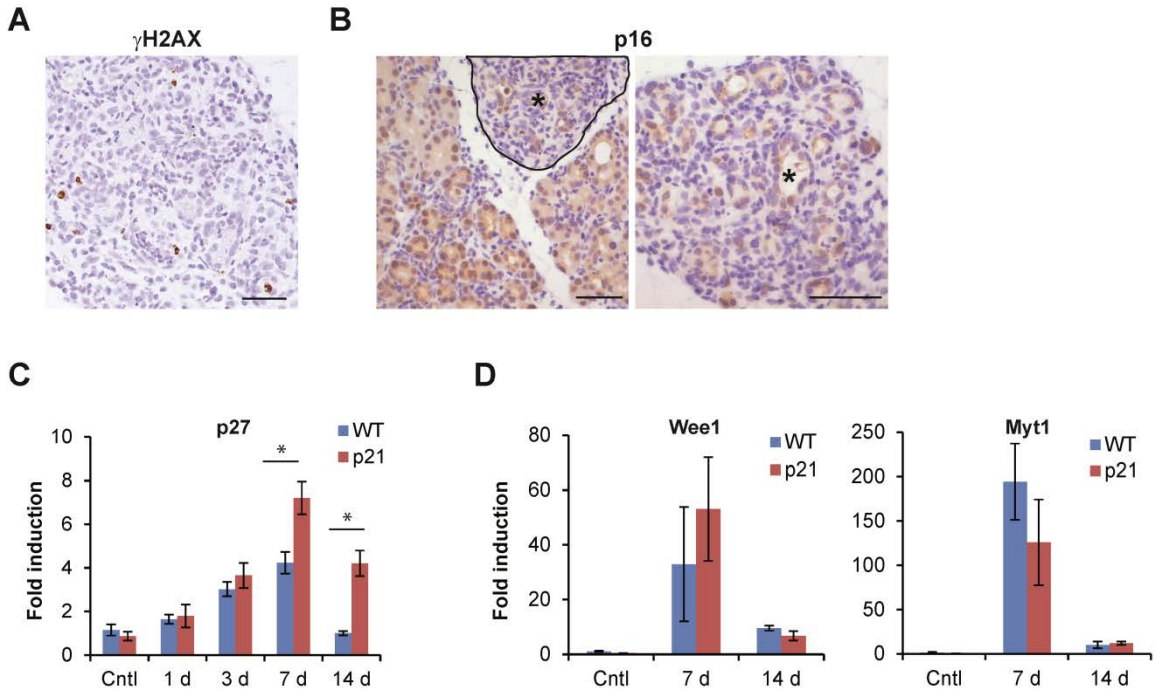

A
γH2AX
B
p16
*
*
C
p27
WT
p21
Fold induction
10
8
6
4
2
0
*
*
Cntl
1 d
3 d
7 d
14 d
D
Wee1
WT
p21
Fold induction
80
60
40
20
0
Cntl
7 d
14 d
Myt1
WT
p21
250
200
150
100
50
0
Cntl
7 d
14 d

**Fig. S9**

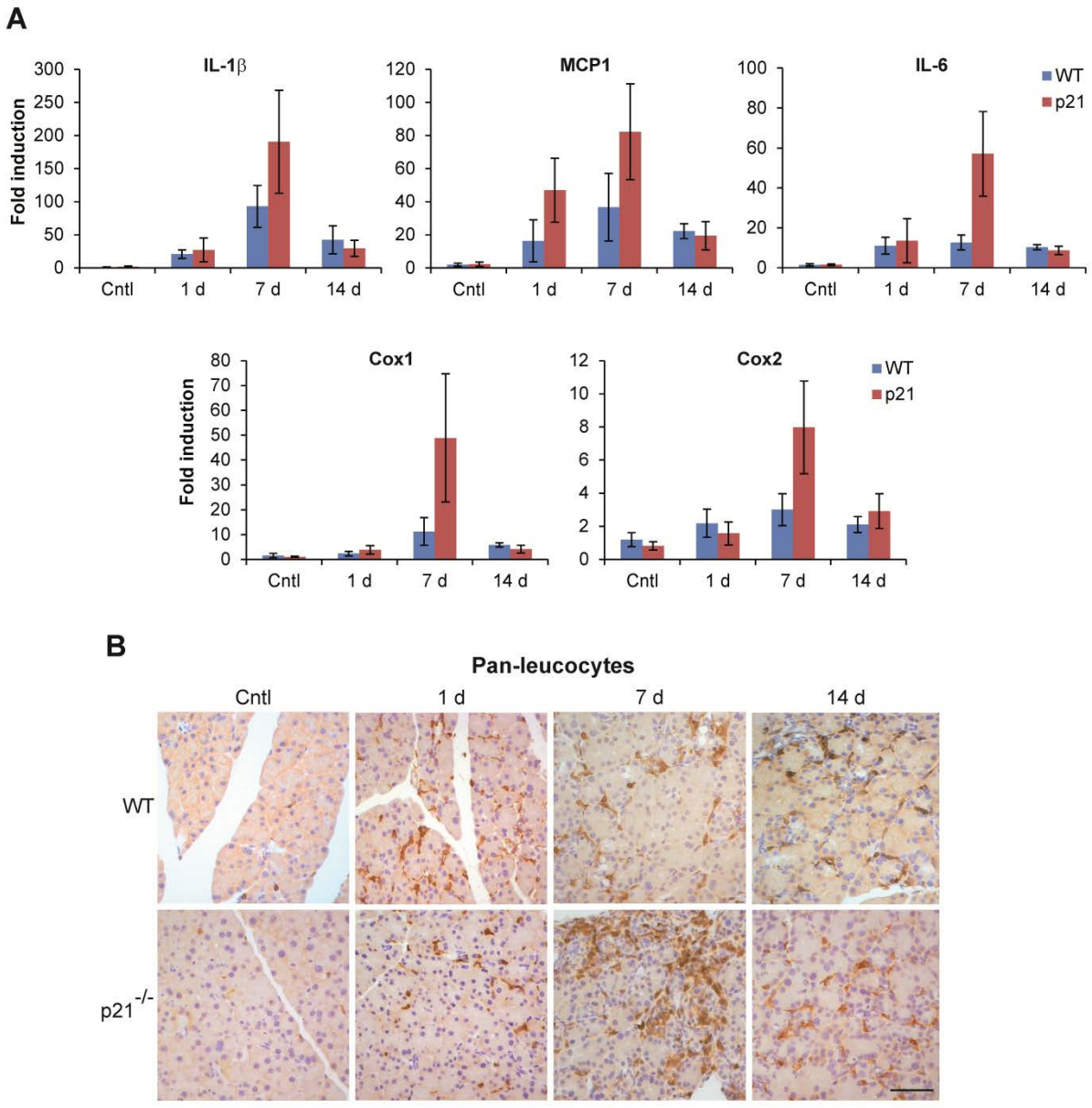

A
IL-1β
MCP1
IL-6
Cox1
Cox2
Fold induction
WT
p21
Cntl
1 d
7 d
14 d
B
Pan-leucocytes
WT
p21-/-

Fig. S10

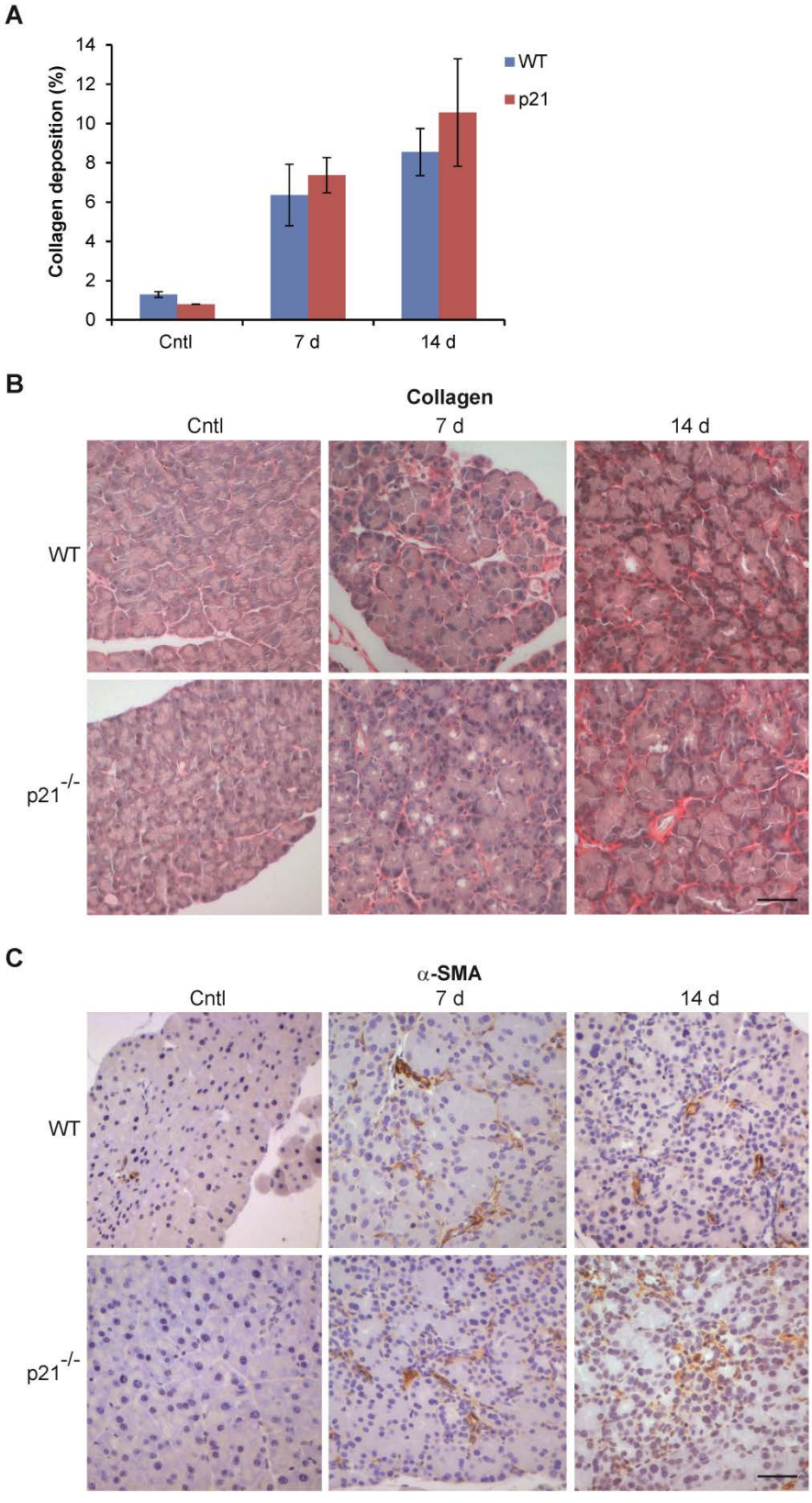

A
Collagen deposition (%)
14
12
10
8
6
4
2
0
Cntl
7 d
14 d
WT
p21
B
Collagen
Cntl
7 d
14 d
WT
p21-/-
C
α-SMA
Cntl
7 d
14 d
WT
p21-/-

**Fig. S11**

**Supplementary Online Material**

## Material and Methods

### *Pancreatitis induction*

Animals received six hourly injections of 50 μg/kg cerulein on Monday, Wednesday and Friday and were harvested on Tuesday after one set of injections (day 1), on Thursday after two sets of injections (day 3) or on Monday after three (day 7) or six (day 14) sets of injections, without receiving cerulein treatment on the same day.

### *Primary acinar and AR42J cell cultures*

Primary acinar cell cultures were prepared according to [1]. Trans-differentiation was induced by addition of 50 ng/ml recombinant hTGF-α (R&D Systems). Cultures were maintained in Waymouths MB 752/1 medium supplemented with 50 U/mL penicillin, and 50 μg/mL streptomycin, 0.1% fetal bovine serum (FBS), 0.1 mg/ml soybean trypsin inhibitor, and 1 μg/ml dexamethasone, with daily medium replacement. AR42J cells were maintained in Kaighn's modified Ham's F-12 medium with 20% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cells were incubated at 37°C in a 5% $CO_2$ atmosphere. Cell death was quantified by trypan blue exclusion. AR42J cells were infected for 24 hours at the multiplicity of infection (MOI) described in the figure legends.

### *Human Samples*

Human pancreas biopsy and serum samples were obtained from the University Hospital in Zurich. All samples were biobank registered and kept anonymous. The research project was authorized by the Ethics Committee of the Canton of Zurich (reference number, StV 26-2005). The study followed the ethical guidelines of the Helsinki declaration.

### *Histology and immunohistochemistry*

Pancreas specimens were embedded in paraffin for histological analyses as described.[2] For immunofluorescence analysis of cultured cell lines, AR42J cells were fixed in 3.6% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Primary antibodies used in this study were: rabbit anti-phospho-histone 3 (Millipore, Massachusetts, USA), rabbit anti-PCNA (Abcam, Cambridge, UK), mouse anti-p21 (BD Pharmingen, NY, USA), rabbit anti-β-catenin (Cell Signaling, Danvers, USA), rabbit anti-cyclin B (Cell Signaling, Danvers, USA), rabbit anti-p27 (Santa Cruz, Santa Cruz, USA), rabbit anti-stat3 (Cell Signaling, Danvers, USA), rabbit anti- Cdc25c-phospho T48 (Abcam, Cambridge, UK), rabbit anti-sox9 (Millipore, Massachusetts, USA), mouse anti-αSMA (Dako, Glostrup, Denmark), rabbit anti-amylase (Sigma-Aldrich, Buchs, Switzerland), rabbit anti-Ki67 (Abcam, Cambridge, UK), rabbit anti-PanKer (Cell Signaling, Danvers, USA), rabbit anti-p48 (gift from Helena Edlund, UMU), rabbit anti-coronin 1 (gift from Jean Pieters), rabbit anti-pu.1 (Cell Signaling, Danvers, USA), rabbit anti-p62/SQSTM1 (MBL, Massachusetts, USA). Secondary antibodies used in this study were: AlexaFluor 594 goat anti-rabbit IgG, AlexaFluor 488 goat anti-rat IgG (Life Technologies, Carlsbad, California, USA). Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI). Detection of DNA fragmentation in apoptotic cells was performed with a TUNEL assay using an ApopTag peroxidase Kit (MP Biomedicals, Illkirch, France). Collagen fibril deposition was detected with Sirius red staining.

Microscopy analyses were performed on a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss Microimaging, Göttingen, Germany) or on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany), using the appropriate settings. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, NL).

### *Biochemical analysis of blood*

For determination of pancreatic enzymes present in the serum, blood was sampled by heart puncture. Blood cells were precipitated by immediate centrifugation and amylase and lipase levels were measured using the Cobas c111 spectrophotometer (Roche Diagnostic GmbH, Mannheim, Germany).

### *Transcript analysis*

The following Taqman probes (Applied Biosystems) were used: p53 (Trp53) Mm01731287_m1, p27 (Cdkn1b) Mm00438168_m1, p15 (Cdkn2b) Mm00483241_m1, p16 (Cdkn2a) Mm00494449_m1, p19 (Cdkn2d) Mm00486943_m1, Mist1 Mm00487695_m1, Sox-9 Mm00448840_m1, Hes-1 Mm01342805_m1, Notch1 Mm00435249_m1, Ccna2 (Cyclin A2) Mm00438064_m1, Ccnb2 (Cyclin B2) Mm01171453_m1, Ccnd1 (Cyclin D1) Mm00432359_m1, Ccne1 (Cyclin E1) Mm00432367_m1, Cdc25a Mm00486880_m1, Cdc25b Mm00499136_m1, Cdc25c Mm00483162_m1, CCKaR Mm00438060_m1, TGFβ1 Mm00441724_m1, TGFβR2 Mm00436977_m1, Wee1 Mm00494175_m1, Pkmyt1 Mm01309244_m1 IL-1b Mm00434228_m1, MCP-1 Mm00441242_m1, IL-6 Mm00446190_m1, cox-1 Mm00477214_m1, cox-2 Mm00478374_m1, amy Mm00651524_m1.

### *Western blotting*

Immunoblotting was performed by homogenizing tissue samples in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim,

Germany). Protein concentrations were determined using a Bradford protein assay (BioRad, Hercules, CA, USA). Aliquots corresponding to 20 or 40 μg of proteins were separated by SDS-PAGE electrophoresis. Blotting and chemiluminescent detection of immunoreactive bands were performed using the V3 Western Workflow system (BioRad, Hercules, CA, USA), according to the manufacturer protocols. Primary antibodies (rabbit anti-sox9 (Millipore, Massachusetts, USA), rabbit anti-amylase, rabbit anti-β-catenin (Cell Signaling, Danvers, USA), rabbit anti-cleaved caspase-3 (Cell Signaling, Danvers, USA), rabbit anti-LC3B (Cell Signaling, Danvers, USA), rabbit anti-β-actin (Sigma-Aldrich, Buchs, Switzerland), rabbit anti-GAPDH (Santa Cruz, Santa Cruz, USA) were incubated overnight at 4°C.

***Statistical analyses***

Groups of 5 animals were tested for each experiment. The data are expressed as the means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test or one-way analysis of variance (GraphPad Prism 4.0c; GraphPad Software, Inc.), and a probability value <0.05 was considered statistically significant. When the overall probability value was <0.05, the Dunnett multiple-comparisons test was used as a post-test to determine whether there was a significant difference between values of control (reference sample) and samples of interest.

**Figure legends**

**Figure S1**. **A**. Immunostaining of p21 in WT mice after 7 days of cerulein treatment. Arrows show interstitial cells with elongated nuclei negative for p21. Incubation with secondary antibody alone was used as a negative control. **B**. qPCR of Mist1 and amylase during the indicated time of cerulein-induced pancreatitis. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

**Figure S2**. Serum levels of amylase (**A)** and lipase **(B)** during cerulein treatment. **C**. qPCR of CCK receptor A during the indicated time of cerulein-induced pancreatitis. **D**. Hematoxylin-Eosin (H&E)-stained sections of pancreata at the indicated time of cerulein treatment. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

**Figure S3**. **A**. TUNEL staining of apoptotic cells during the indicated time of cerulein-induced pancreatitis. Immunoblotting for cleaved caspase 3 (**B**) and LC3-II (**C**) at the indicated time of pancreatitis induction. 40 μg proteins were processed for Western blotting and band intensity was normalized using GAPDH as a loading control. **D**. Immunostaining of the autophagic marker p62. **E**. Pan-leucocyte immunostaining with anti-coronin-1. **F**. Quantification of PU.1 positive myeloid cells, including neutrophils and macrophages, at the indicated time of cerulein-induced pancreatitis. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

**Figure S4**. **A**. Immunostaining of p21 in WT and $p21^{-/-}$ mice after 7 days of cerulein treatment. I, islet. **B**. Immunostaining of Sox9, αSMA and pan-leucocytes in serial sections from WT and $p21^{-/-}$ mice after 7 days of cerulein treatment. **C**. qPCR of progenitor-cell markers during the indicated times of cerulein-induced pancreatitis. **D**. Immunostaining (upper panel) and quantification of Sox9 in acinar (Ac) and interstitial (Int) cells (lower panel) showed a tendency of up-regulation of the protein in intact acini in $p21^{-/-}$ mice. Results are average ± SEM (n=5), *p<0.05.

**Figure S5**. **A**. qPCR of Cdc25B and Cdc25A during cerulein treatment. Results are average ± SEM (n=5). **B**. Co-localization analysis of Ki67 and p21 in the nuclei of WT pancreata after 7 days of cerulein treatment. Arrows indicate nuclei in the replicative state expressing p21. **C**. Co-localization analysis of amylase and p21 in WT pancreata after 7 days of cerulein treatment. Arrows indicate acinar cell expression of p21. Scale bars: 30 μM.

**Figure S6**. **A**. Immunostaining of Ki67, PCNA and phospho-histone 3 (pH3) during cerulein treatment. **B**. Quantification of pH3 positive pancreatic acinar (Ac) and interstitial (Int) cells showed comparable proliferation rate in WT and $p21^{-/-}$ mice. Scale bars: 50 μM.

**Figure S7**. **A**. Hematoxilin/eosin (H&E) and Masson's trichrome staining of WT and $p21^{-/-}$ pancreata after a week of recovery following one week of cerulein treatment. Quantification of amylase activity in pancreatic tissues (**B**) and sera (**C**) of WT and $p21^{-/-}$ mice after 7 days of recovery time. **D**. Immunostaining of the autophagy marker p62 in WT and $p21^{-/-}$ pancreata after a week of recovery following one week of cerulein treatment. Results are average ± SEM (n>5), *p<0.05. Scale bars: 30 μM.

**Figure S8**. **A**. Immunolocalization of β-catenin and p21 in AR42J cells. Nuclei are stained with DAPI. **B**. Left panel, immunostaining of p21 (upper row) or live imaging (lower row) of AR42J cells infected with adenoviruses expressing p21 (Adp21) or GFP (AdGFP). Nuclei are stained with DAPI (blue). Note the nuclear and cytosolic localization of p21 and GFP, respectively. Right panel, densitometric quantification after Western blotting of β-catenin /GAPDH levels following incubation for 24 h with Adp21 or AdGFP. Results are average ± SEM (n=3). **C**. Quantification of trypan blue–positive cells after infection with Adp21 at the indicated multiplicity of infection (MOI). AdGFP at 100 MOI was used as control. Data are average of percentage of total cell number (TOT) ± SEM (n=3). **D**. qPCR of p53 in WT and $p21^{-/-}$ mice during

cerulein treatment. Results are average ± SEM (n=5). Scale bars: 50 μM.

**Figure S9**. **A**. γH2AX immunostaining in ADM areas of $p21^{-/-}$ mice after 7 days of cerulein treatment. **B**. Immunostaining of p16 showing expression of the protein in intact acini of $p21^{-/-}$ mice after 7 days of cerulein treatment. Asterisks indicate ADM areas. **C**. qPCR showed increased expression of p27 transcripts in $p21^{-/-}$ mice after 7 days of cerulein treatment. **D**. qPCR of cyclin B/Cdk1 inhibitors Wee1 and Myt1 during cerulein treatment. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

**Figure S10**. Inflammatory response is stimulated in the absence of p21. qPCR of pro-inflammatory markers (**A**) and pan-leucocyte immunostaining with anti-coronin-1 (**B**) showed increased inflammation in $p21^{-/-}$ mice 7 days after induction of pancreatitis. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μM.

**Figure S11**. **A**, **B**. Quantification of Sirius red staining, expressed as percentage of total field area, showed comparable collagen deposition in WT and $p21^{-/-}$ mice. **C**. Similarly, immunostaining of α-SMA showed comparable activation of stellate cells in the two mouse strains. Results are average ± SEM (n=5). Scale bars: 50 μM.


1. Algul H, Wagner M, Lesina M*, et al.* Overexpression of ErbB2 in the exocrine pancreas induces an inflammatory response but not increased proliferation. *Int J Cancer* 2007; **121**: 1410-1416.

2. Silva A, Weber A, Bain M*, et al.* COX-2 is not required for the development of murine chronic pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G968-975.

# 5. Manuscript B

## Inactivation of TGF-β receptor signalling in epithelial cells promotes acinar replication and acinar-to-ductal metaplasia formation during pancreatitis

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**Contribution:**

This study represents a part of my work done during the third and fourth years of the PhD. All experimental work was done by me, excluding the generation of transgenic mouse lines. I also contributed to drafting/revising of large parts of the manuscript.

**Inactivation of TGF-β receptor II signaling in pancreatic epithelial cells promotes acinar cell proliferation and acinar-to-ductal metaplasia formation during pancreatitis**

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**Running title:** TGFβ and pancreatitis

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**Competing interests:** none

**Word count:** 4000

## Abstract

Determination of signaling pathways that regulate pancreatic regeneration following pancreatitis is critical for implementing therapeutic interventions. In this study we elucidated the molecular mechanisms underlying the effects of transforming growth factor-β (TGF-β) in pancreatic epithelial cells during tissue regeneration. To this aim, we conditionally inactivated TGF-β receptor II (TGF-β RII) using a Cre/loxP system, under control of pancreas transcription factor 1a (PTF1a) promoter specific for the pancreatic epithelium, and evaluated the molecular and cellular changes in a mouse model of cerulein-induced pancreatitis. We showed that TGF-β RII signaling does not mediate the initial acinar cell damage observed at the onset of pancreatitis. However, we found that TGF-β RII signaling limits both acinar cell replication and ADM formation during the regenerative phase of the disease. Analyses of molecular mechanisms underlying the observed phenotype revealed that TGF-β RII signaling stimulates the expression of the cyclin-dependent kinase inhibitor p16 and intersects with the EGFR signaling axis. Finally, TGF-β RII ablation in epithelial cells resulted in increased infiltration of inflammatory cells in the early phases of pancreatitis and increased activation of pancreatic stellate cells in the later stages of pancreatitis, thus highlighting a TGF-β-based cross talk between epithelial and stromal cells regulating the development of pancreatic inflammation and fibrosis. Collectively, our data not only contribute to clarify the cellular processes governing pancreatic tissue regeneration, but also emphasize the conserved role that TGF-β plays as a tumor suppressor both in the regenerative process following pancreatitis and in the initial phases of pancreatic cancer.

## Introduction

Repair of pancreatic tissue is observed following pancreatitis, an acute or chronic inflammation of the exocrine pancreas that continues to be a clinical challenge with potentially lethal outcome [1]. Transforming growth factor-β (TGF-β) plays an essential role in tissue repair and elevated expression of this molecule has been documented in human pancreatic tissue samples during pancreatitis [2, 3]. In addition, studies using rodent models of the disease demonstrated that TGF-β regulates tissue repair by promoting extracellular matrix remodeling and fibrogenesis [4, 5, 6, 7, 8, 9], thus causally linking TGF-β signalling to the pathophysiology of pancreatitis.

TGF-β receptor II (TGF-β RII) is a central component of the TGF-β signaling pathway, which couples ligand binding to downstream activation of intracellular Smads and transcription of target genes (recently reviewed in [10]). In previous studies, the role of TGF-β RII in the development of pancreatitis was investigated by expressing a dominant-negative mutant form of the receptor under control of metallothionein 1 (MT1) [11, 12]

or pS2 mouse trefoil peptide promoters [13]. However, these transgenic models delivered conflicting results regarding the role of TGF-β signaling in the development of pancreatitis. Furthermore, these mice exhibited immunological and molecular alterations in the pancreas under untreated control conditions, suggesting that the overexpression of dominant-negative TGF-β RII may also affect the functionality of non-epithelial pancreatic cells, including immune and stromal cells.

In this study, we aimed to clarify in more detail how TGF-β signaling elicited in pancreatic epithelial cells contributes to three key phases of pancreatitis, namely i) onset of the initial acute inflammation, ii) proliferation of acinar cells and iii) development of tissue fibrosis. To overcome the limitations associated with the expression of dominant-negative TGF-β RII, we generated a conditional knockout allele of the receptor by crossing *TGF-β RII*$^{lox}$ homozygous mice [14] to *Ptf1a-Cre* transgenic animals. This transgenic model exhibits functional inactivation of TGF-β signaling in pancreatic epithelial cells, without the molecular alterations observed in the previous dominant negative models. As a result, this model allows us to dissect the function of TGF-β signalling in these cell types during the distinct phases of the disease.

## Material and methods

### *Animal experiments*

All animal experiments were performed in accordance with Swiss federal animal regulations and approved by the cantonal veterinary office of Zurich. Transgenic mice expressing TGF-β receptor II$^{flox/flox}$ [15] and mice harboring pancreas transcription factor 1a (PTF1a) promoter cre transgene (PTF1a$^{cre}$) (MMRRC, USA) were bred to generate PTF1a$^{cre}$/TGF-β RII $^{flox/flox}$ (TGF-β RII KO) mice. The CRE-negative littermates were used as controls and showed no difference from TGF-β RII $^{flox/flox}$ mice (not shown). Pancreatitis was induced in adult (8-10 weeks of age) male mice via six intra peritoneal (i.p.) injections of 50 μg/kg cerulein administered hourly every second day over a two week period, as previously described [16]. Control animals received 0.9% NaCl injections.

### *Histology, immunohistochemistry and immunoblotting*

Detailed protocols and primary antibodies used in this study are listed in Supplementary Materials and Methods. Quantification of labelled cells was performed in at least 10 randomly selected high-power fields (×100) per slide. Non-acinar tissue areas (islets, vessels, fibrotic tissue) were excluded from the analysis.

### *Transcript analysis*

Total RNA was extracted from pancreata as previously described [17]. Transcript levels were normalized using 18S rRNA as a reference and expressed as fold regulation relative to the value of untreated control animals, set as one. Taqman probes (Applied Biosystems) used in this study are listed in Supplementary Materials and Methods.

## Results

### Conditional TGFβRII KO mice show normal pancreatic development and function

*Ptf1a*$^{cre/+}$*;Tgf-β RII*$^{flox/flox}$ mice with selective TGF-β RII ablation in pancreatic epithelial cells (designated as TGF-β RII KO) were born at the expected frequency, similarly to control *Ptf1a*$^{+/+}$*;Tgf-β RII*$^{flox/flox}$ animals (designed as control) or *Ptf1a*$^{cre/+}$*;Tgf-β RII*$^{+/+}$. Confirmation of TGF-β RII deletion was obtained by PCR on whole pancreas using a primer pair designed according to [15] to amplify a 692 base pair fragment after successful recombination (Fig. 1A). Eight week old TGF-β RII KO mice showed normal pancreatic histology, with acinar architecture (Fig. 1B) and amylase content (Fig. 1C) comparable to control animals. *Ptf1a*-driven Cre recombination results in knock out of TGF-β RII also in pancreatic islets [18]. No abnormalities were observed in these structures and endocrine parameters including islet size (Fig. 1D), insulin-containing β cells (Fig. S1A) and serum levels of glucose (Fig. 1E) were comparable to control mice. In addition, TGF-β RII KO mice monitored for 18 months did not show morphological alterations in the pancreas [19]. Of note, mice expressing a dominant negative form of TGF-β RII under the control of a methallothionein or pS2 promoters showed higher levels of TGFβ isoform 1 and 3 [11, 12], inflammatory cells [11] and pro-inflammatory cytokines [13] than control mice. Contrary to these results, the described parameters were comparable in the pancreas of control and TGF-β RII KO mice, as assessed by quantification of TGF-β isoform levels (Fig. 1F), pan-leukocyte staining (Fig. 1G) and expression of pro-inflammatory cytokines INF-γ, IL-6 and macrophage marker F4/80 (Fig. 1H, S1B). Taken together, these data indicate that lack of TGF-β RII in pancreatic epithelia does not compromise normal pancreatic development and physiology.

### Conditional TGF-β RII KO mice show comparable enzymemia but increased inflammatory infiltrates following cerulein-induced pancreatitis

The role of TGF-β signalling during the acute phase of pancreatic inflammation is still

controversial, as pancreatic overexpression of a dominant negative form of TGF-β RII has been reported to either exacerbate [13] or attenuate [11] cerulein-induced acute pancreatitis, reflected by increased or decreased serum levels of amylase and lipase, respectively. To clarify these contradictory results, we evaluated the severity of acute pancreatitis in control and TGF-β RII KO mice. Following cerulein treatment, the two strains displayed similar pancreatic morphology (Fig. 2A) and comparable serum levels of amylase and lipase, early indicators of acinar cell damage (Fig. 2B). Serum levels of liver alanine aminotransferase, which are reported to slightly increase after cerulein treatment [20], were also comparable in the two strains (Fig. 2C). In addition, acinar trypsinogen was activated following pancreatitis and the level of activation was not statistically different in the two strains, even if TGF-β RII KO mice showed a trend of lower activation compared with the control animals (Fig. 2D). TGF-β RII KO mice had more apoptotic acinar cells than control animals 8 hours after treatment (Fig. 2E), but the number of apoptotic cells accounted for less than two percent of the total number of acinar cells (Fig. S2A), indicating that apoptosis is not greatly induced in the absence of TGF-β signalling. The attenuation of cerulein-induced pancreatitis reported in mice with dominant negative TGF-β RII expression was linked to CCK/cerulein resistance and up-regulation of CCK-A receptor both in untreated condition and upon cerulein stimulation [11]. Contrary to these results, TGF-β RII KO and control mice showed comparable levels of CCK-A (Fig. 2F) and B (Fig. S2B) receptors both in untreated conditions and following cerulein treatment, thus indicating that TGF-β signaling is not required to modulate mRNA expression of CCK-A/B receptors. In addition, the previously described mice expressing dominant negative TGF-β RII showed higher levels of pancreatic inflammation under untreated condition. However, inflammation levels did not further increase during the development of pancreatitis and were similar to the increased levels observed in control mice after cerulein treatment [11, 13]. On the contrary, despite the comparable enzymemia and tissue morphology observed in control and TGF-β RII KO mice, immunostaining showed more infiltration of leukocytes (Fig. 2G) and macrophages (Fig. S2C) in TGF-β RII KO one day after the beginning of cerulein treatment. Similarly, enumeration of PU.1 positive myeloid cells, including neutrophils and macrophages, confirmed the increased infiltration in the first day of treatment, while infiltrate levels and cytokine expression were comparable in the two strains at the later time points of the treatment (Fig. 2H, S2D). These data indicate that lack of TGF-β signalling in epithelial cells did not exacerbate acinar cell damage during cerulein treatment. However, it resulted in a transient increase in immune cell infiltration during the acute phase of pancreatitis.

**Conditional TGF-β RII KO mice show increased cell cycle entry following cerulein-induced pancreatitis**

TGF-β is a negative regulator of cell cycle progression inducing cell cycle arrest in both $G_0/G_1$ and $G_2$/M phases [21]. As TGF-β RII is up-regulated in the pancreas of control mice during pancreatitis (Fig. S3A), we evaluated whether lack of TGF-β RII in epithelial cells was associated with increased levels of acinar replication following induction of the disease. Ablation of canonical TGF-β signalling in TGF-β RII KO mice was further confirmed by reduced phosphorylation of Smad2 and Smad3, which are triggered downstream of TGF-β RII activation (Fig. S3B). In untreated conditions, TGF-β RII KO mice did not show spontaneous activation of the cell cycle in epithelial cells (Fig. 3A). However, after one week of cerulein treatment, these mice had more replicating acinar cells than control animals, as shown by Ki67 and Bromodeoxyuridine (BrdU) staining (Fig. 3A, S3C). The mitotic marker phosphor-histone 3 (pH3) showed increased expression but without reaching statistical significance (Fig. S3C). Importantly, the number of replicating interstitial cells increased in a comparable manner in the two strains (Fig. S3D), while cerulein treatment did not induce proliferation of ductal and islet cells (Fig. S3D), indicating that ablation of TGF-β RII in pancreatic epithelia increased cell cycle entry exclusively in acinar cells following induction of pancreatitis.

TGF-β signalling can limit cell cycle progression by inducing the expression of cyclin-dependent kinase (Cdk) inhibitors [22]. We then tested whether increased acinar replication in TGF-β RII KO mice was associated with lower Cdk inhibitor expression. Indeed, after one week of cerulein treatment, TGF-β RII KO pancreata showed lower expression of p16 in the nuclei of acinar cells, while the staining in interstitial cells was similar in the two strains (Fig. 3B). A trend of lower p16 levels was also evident at the transcript level (Fig. S4). Expression of p21 and p27 was comparable in the two mouse strains (Fig. S3F). TGF-β signalling can also limit cell cycle progression by inducing apoptosis (reviewed in [23]). However, the number of TUNEL positive apoptotic cells was comparable in both strains after three and seven days of cerulein treatment (Fig. 3C), suggesting that the increased replication observed in the absence of TGF-β RII is not derived from decreased apoptosis of acinar cells, but rather from limited expression of the cyclin-dependent kinase inhibitor p16.

**Conditional TGF-β RII KO mice show increased ADM formation following cerulein-induced pancreatitis**

Next, we evaluated whether lack of TGF-β signalling in epithelial cells affects the formation of acinar-to-ductal metaplasia (ADM), a transient trans-differentiation of acinar cells observed during pancreatic regeneration. The percentage of mice showing ADM formation was higher in TGF-β RII KO mice after three and seven days of cerulein treatment (Fig. 4A) and the lesions were bigger three days after the treatment (Fig. 4B). ADM lesions showed down-regulation of amylase content and higher number of apoptotic cells compared with intact acini, while the level of Ki67 positive cells was similar to the surrounding intact tissue (Fig. 4C). As we detected increased ADM formation in TGF-β RII KO mice, we investigated whether factors promoting ADM are activated in the absence of epithelial TGF-β signalling. Cells undergoing ADM in TGF-β RII KO mice showed high levels of active phosphorylated epidermal growth factor receptor (EGFR) (Fig. 4D), a critical inducer of ADM formation [24]. Altogether, these results indicate that TGF-β signalling in epithelial cells limits ADM formation following cerulein-induced pancreatitis.

**Lack of TGF-β RII results in increased pancreatic fibrosis**

Several reports highlighted the importance of TGF-β signalling for the development of fibrosis in the pancreas (reviewed in [6]). However, some controversy still remains, as pancreatic expression of dominant negative TGF-β RII under control of the MT1 or pS2/TFF1 promoters either increased [12] or reduced [9] pancreatic fibrosis, respectively. Thus, we assessed the level of fibrosis in TGF-β RII KO animals during pancreatitis. TGF-β RII KO pancreata showed higher expression levels of αSMA (Fig. 5A) and enhanced Sirius red staining (Fig. 5B), which indicate increased activation of stellate cells and collagen deposition in these mice. To understand the mechanism by which ablation of TGF-β signalling in epithelial cells led to increased activation of stromal stellate cells, we evaluated whether the absence of TGF-β RII resulted in a compensatory increase of TGF-β levels, a potent activator of stellate cells. Quantification of TGF-β in pancreata, by qPCR, (Fig. S5A) and western blotting (Fig. S5B), and in blood, by ELISA assay (Fig. S5C), showed comparable levels of TGF-β in the two strains, indicating that the increased fibrosis observed in TGF-β RII KO mice is not a consequence of increased TGF-β expression. Then we tested whether TGF-β activators increased in TGF-β KO RII mice. An important activator of TGF-β is the $\alpha_v$ subunit of transmembrane integrins that binds and activates latent TGF-β stored in the extracellular matrix (recently reviewed in [25]). Our analyses focussed on the $\alpha_v\beta_6$ isoform, as its deletion or pharmacological inhibition was sufficient to prevent latent TGF-β1 activation in pancreas [26] and lung [27, 28]. $\alpha_v\beta_6$ integrin was up-regulated during the development of pancreatitis and was localized on acinar cell membrane and ADM areas (Fig.5C). However, its expression was similar in control and TGF-β RII KO mice, indicating that lack of TGF-β receptor does not result in compensatory up-regulation of $\alpha_v\beta_6$ integrin and increased TGF-β activation. Finally, we analyzed whether pro-fibrotic cytokines [29] were elevated in TGF-β RII KO mice. We did not observe any difference in the transcript levels of CXCL1, CXCL2, CXCL16 and IGF-1 (Fig. S6). Collectively, these data indicate that lack of TGF-β receptor in pancreatic epithelial cells is sufficient to increase the fibrotic response during pancreatitis without increasing the expression of its ligand TGF-β or pro-fibrotic factors.

**Discussion**

In this study, we demonstrated that TGF-β signaling in epithelial cells is dispensable for the development of the adult pancreas. During onset of acute pancreatitis, this pathway does not appear to influence acinar damage, while in the progression of the disease it is required to constrain acinar cell replication and development of acinar-to-ductal metaplasia.

The main advantage of our TGF-β RII knock-out model, compared with the overexpression of a dominant negative TGF-β RII mutants used previously [11, 12, 13], consists in the absence of immunological and molecular alterations under untreated conditions. This different phenotype can be explained by the specificity of the promoters used as the *Ptf1a-Cre* transgene is selective for the pancreas, while MT1 or pS2 promoters are active also in extra-pancreatic tissues [12, 13] and may affect systemic responses. Furthermore, non-physiological gain-of-function activities elicited by ectopically overexpressed proteins may contribute to the baseline alterations observed in the previous studies. Thus, our TGF-β RII KO model with normal phenotype under untreated conditions allows the characterization of TGF-β signaling during pancreatitis induced upon cerulein treatment.

**TGF-β receptor II and acute pancreatitis**

The role of TGF-β receptor in the early phases of cerulein-induced pancreatitis has been examined in two previous studies using dominant negative mutants of the TGF-β receptor II under control of MT1 or pS2 promoters. These reports reached opposite conclusions on the role of TGF-β

receptor, as its dominant negative form either exacerbated [13] or reduced [11] the severity of pancreatitis. Importantly, despite the opposite disease outcome, even under untreated conditions both these mutants showed higher levels of pancreatic inflammation, which did not further increase following cerulein treatment. As discussed in these studies, the presence of inflammatory infiltrates under untreated conditions may be attributable to the presence of mutant TGF-β receptor II not only in acinar cells but also in inflammatory cells. Thus, these models are not suitable to dissect the selective contribution of TGF-β signaling derived from parenchymal and inflammatory cell types. Nevertheless, these studies highlight the intriguing concept that the level of inflammation *per se* does not directly correlate with the damage of acinar cells and the severity of pancreatitis. In our TGF-β RII KO model we observed that the most sensitive parameter to determine acinar cell damage, namely, release of amylase and lipase into the blood, was comparable to control animals. Likewise, the two strains showed similar expression of CCK receptors and morphological appearance, suggesting that TGF-β signalling in epithelial cells does not regulate the severity of acinar cell damage. However, we observed increased numbers of infiltrating cells in TGF-β RII KO mice one day after induction of pancreatitis. This apparent conundrum of an effect observed in non-parenchymal inflammatory cells following ablation of TGF-β receptor II in parenchymal cells is similar to what we observed in regards to development of fibrosis at later stages of the disease. For this reason, we will discuss the potential mechanisms underlying these phenotypes in the paragraph dedicated to fibrosis.

**TGF-β receptor II and pancreatic regeneration**

Earlier reports showed that acinar cell replication is inhibited by TGF-β overexpression in the pancreas or by TGF-β administration to pancreatic explants [4, 30, 31, 32], indicating that acinar cells are responsive to TGF-β signalling. Further studies showed that overexpression of a dominant negative TGF-β RII in the pancreas is sufficient to increase the proliferation of acinar cells in the absence of a regenerative stimulus [12]. However, this study was complicated by the fact that the transgenic mice presented spontaneous inflammation and elevated TGF-β levels [11, 12], thus raising the question whether the increased acinar replication could have been triggered by factors released by TGF-β-stimulated non-acinar cells. Indeed, acinar replication was not increased in our TGF-β RII KO untreated mice, which did not have increased inflammation or TGF-β levels. This suggests that TGF-β RII signalling is not required for homeostatic turnover of acinar cells in the absence of cell injury. Following pancreatitis, on the other hand, absence of TGF-β RII resulted in an increased rate of replication exclusively in acinar and not in other epithelial or interstitial cells, suggesting a specific inhibition of acinar replication upon an inflammatory insult via TGF-β RII signalling via up-regulation of the cyclin-dependent kinase inhibitor p16. This is in marked contrast to what observed in pancreatic β-cell where TGF-β RII signaling promoted β-cell replication following pancreatic duct ligation [18]. Conversely, the same authors and [33] showed that TGF-β RII signaling inhibited β-cell replication following partial pancreatectomy, further supporting the concept that TGF-β regulates cellular replication in a cell type and context dependent manner. These alternating growth-inhibitory and growth-promoting effects of TGF-β are of particular importance during pancreatic tumorigenesis where TGF-β converts over time from a tumor suppressor to a mitogen that enhances cancer progression [34, 35].

Of note, we found that the number of acinar cells positive for phosphor-histone 3, a marker of M phase not evaluated in previous studies, was comparable in the two strains. This suggests that, despite increased cell cycle entry in the absence of TGF-β RII, compensatory mechanisms may be activated to limit the completion of acinar cell division.

In addition, we observed that lack of TGF-β RII was also associated with increased formation of ADM during the course of pancreatitis. Spontaneous ADM formation was also observed in mice expressing a dominant negative TGF-β RII [12] and in mice with disrupted TGF-β signaling following overexpression of the inhibitory Smad7 under control of an elastase promoter [36]. Unexpectedly, Smad7 overexpression reduced ADM formation triggered by cerulein-induced pancreatitis [37]. While the molecular mechanisms responsible for these discordant phenotypes are not clear, they may result from context-dependent integration of different signaling pathways, as Smad7 inhibits both the TGF-β and the BMP branches of the TGF-β family signaling pathways.

Our analyses elucidated the molecular mechanisms that are regulated *in vivo* by TGF-β signalling during the course of pancreatitis. Specifically, we found increased activation of EGFR, a potent inducer of ADM formation and malignant transformation [38], in the absence of TGF-β RII, suggesting that TGF-β exerts an inhibitory effect on the EGFR signaling in acinar cells. A similar antagonistic role of TGF-β RII and EGFR signalling has recently been reported in regulating the replication of β-cells following pancreatic duct ligation [39], raising the intriguing possibility that a TGF-β/EGFR cross-talk is

intrinsic to pancreatic regeneration. The question then arises as to whether the observed TGF-β intersection with the EGFR signalling is at the base of the tumor suppressor role of TGF-β in pancreatic cancer. While further studies are required to unequivocally demonstrate this concept *in vivo*, previous reports showed that ablation of TGF-β signalling by loss of Smad4 increased the expression of EGFR in pancreatic cancer cells *in vitro* [40, 41]. In addition, loss of TGF-β RII was associated with higher EGFR levels and more aggressive and metastatic tumors in pancreatic ductal adenocarcinoma [19] and head-and-neck squamous cell carcinoma *in vivo* [42]. Thus, it is tempting to speculate that TGF-β-mediated inhibition of EGFR signaling may be an early conserved molecular mechanism to counteract tumorigenesis, both during regeneration and malignant transformation. Conversely, at later stages of cancer progression, TGF-β/EGFR signaling can develop in a synergistic mode, as previously shown [43, 44, 45], and thus contribute to the tumor supporting function of TGF-β.

**TGF-β receptor II and development of fibrosis**

An unexpected result of our study was the increased collagen deposition in TGF-β RII KO mice. The elevated expression of αSMA suggests that stellate cell activation into fibrogenic myofibroblasts actively contributes to the enhanced stromal reaction. This phenotype observed in non-parenchymal cells following ablation of TGF-β receptor II in parenchymal cells resembles the increased inflammation detected during the acute phase of pancreatitis and suggests the existence of a complex and tightly regulated balance between epithelial and non-epithelial cells during the development of pancreatitis. To identify the molecular mechanisms at the base of the increased inflammation and fibrosis, we evaluated whether ablation of TGF-β receptor resulted in a compensatory increase of TGF-β, as increased TGF-β levels were reported in mice expressing a dominant negative TGF-β RII [12]. TGF-β, acting together with other co-stimulatory molecules, is a very potent regulator of both immune response and fibrogenic processes. In particular, TGF-β1 stimulates chemotaxis of monocytes and neutrophils, the major leukocyte populations that are recruited in the pancreas during pancreatitis. While exerting a pro-inflammatory function in monocytes, TGF-β1 has also an anti-inflammatory effect in monocytes after their differentiation into macrophages. This, together with similar effects on other inflammatory cell types (reviewed in [46]), support a dual role of TGF-β1 in the control of inflammation, namely via promoting the initiation of the process and contributing to its resolution. This time-dependent regulatory activity of TGF-β1 would be consistent with what we observed in TGF-β RII KO mice where the increased inflammation was transient and limited to the first day following induction of pancreatitis. In addition, TGF-β is a potent activator of stellate cells, key players in pancreatic fibrosis following pancreatitis (recently reviewed in [47]). Thus, increased levels of TGF-β could explain the increased inflammatory and fibrotic processes observed in TGF-β RII KO mice. However, quantitative analyses of pancreatic and circulating levels of TGF-β showed similar expression in the two strains. This indicates that increased inflammation and fibrosis do not result from increased TGF-β production and that ablation of TGF-β receptor in epithelial cells does not result in a compensatory up-regulation of its ligand. In this context, an additional aspect of TGF-β regulation can be extrapolated from our results. TGF-β was shown to induce a positive feedback loop in acinar cells by stimulating both TGF-β and TGF-β RII expression [31]. The fact that ablation of epithelial TGF-β RII did not change TGF-β levels *in vivo* suggests that the contribution of epithelial cells to produce TGF-β in the pancreas following cerulein-induced pancreatitis is limited. An alternative mechanism responsible for the observed increased fibrosis is the secretion by epithelial cells lacking TGF-β RII of pro-fibrotic factors normally down-regulated by TGF-β signaling. While seemingly contradictory to the pro-fibrotic function of TGF-β, a similar regulation has been shown in a transgenic model of pancreatic cancer, where TGF-β down-regulated the production of pro-fibrotic CXC chemokines in epithelial cells [48]. However, pro-fibrotic CXC chemokines were not up-regulated in TGF-β RII KO mice, suggesting that this pathway is not likely to contribute to the observed phenotype. Finally, an intriguing explanation for the increased inflammation and fibrosis in TGF-β RII KO mice is that, despite the unchanged levels of TGF-β in the pancreas, this cytokine does not bind to epithelial cells due to the lack of its receptor and consequently it becomes more available to inflammatory and stromal cells, resulting in their increased stimulation. Thus, the ability of epithelial cells to act as a buffer by binding selected cytokines and therefore modulating their availability to non-epithelial cells is likely to be a critical element in the complex network regulating the progression of pancreatic diseases.

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**Contributors**

The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript. KG, performing experiments, generation and analysis of data, writing the manuscript; ES, AZ, CF performing experiments, generation and analysis of data, TR, generation of transgenic lines; SS, study design, writing the manuscript; RG, study design, revising the manuscript. All authors approved the submitted version.

**Data sharing statement** No additional data

**Figure legends**

**Figure 1**. Pancreatic development is normal in TGF-β RII KO mice. **A**. Schematic representation of the breeding strategy to produce mice with TGF-β RII deletion in pancreatic epithelial cells. *Ptf1a*$^{cre/+}$ mice were bred with *Tgf-βRII*$^{flox/flox}$ to generate *Ptf1a*$^{cre/+}$*;Tgf-βRII*$^{flox/flox}$ mice, named TGF-β RII KO. Activation of Cre recombinase in PTF1a positive pancreatic cells removes exon 4 in TGF-β RII, which is required for recruitment of and dimerization with TGF-β RI and consequent normal receptor functions. For validation of TGF-β RII receptor ablation a primer pair was designed to amplify a 692 bp fragment after recombination and removal of exon 4 from the TGF-β RII locus. RT-PCR was performed on whole pancreas samples. **B**. Hematoxylin-Eosin (H&E)-stained sections of untreated control (Cntl) and TGF-β RII KO (KO) pancreata showing comparable tissue morphology. **C**. Amylase activity in untreated pancreatic tissue. **D**. Islet size, expressed as area, in untreated pancreatic tissue. **E**. Blood glucose levels in untreated pancreatic tissue. **F**. qPCR of TGF-β isoforms in untreated pancreatic tissue. **G**. Immunostaining of pan-leukocytes in untreated pancreatic tissue. **H**. qPCR of interferon-γ (INF-γ), interleukin 6 (IL6) and macrophage marker F4/80 in untreated pancreatic tissue. Results are average ± SEM (n≥5). Scale bars: 50 μM.

**Figure 2**. Onset of pancreatitis in TGF-β RII KO mice. **A**. Pancreatitis was induced in control (Cntl) and TGF-β RII KO (KO) mice with six injections of 50 μg/kg cerulein administered hourly on alternate days over a period of one week. The indicated times of animal harvest refer to the first cerulein injection. Hematoxylin-Eosin (H&E)-stained sections of pancreata showing comparable tissue morphology after 8 hours and one day of pancreatitis induction. **B**. Serum levels of amylase and lipase following cerulein treatment. **C**. Serum levels of alanine aminotransferase (ALT) following cerulein treatment. **D**. Quantification of trypsinogen activation following cerulein treatment. **E**. TUNEL staining showing the number of apoptotic cells in intact acini following cerulein treatment. **F**. qPCR of CCK receptors A and B following cerulein treatment. **G**. Immunostaining of pan-leukocytes after one day of cerulein treatment. **H**. Quantification of PU.1 positive myeloid cells infiltrating the pancreas following cerulein treatment. Results are average ± SEM (n≥5), *p<0.05. Scale bars: 50 μM.

**Figure 3**. Acinar cell proliferation does not increase in the absence of TGF-β RII. **A**. Immunostaining (left panel) and quantification (right panel) of Ki67 positive pancreatic acinar cells showed increased proliferation rates in TGF-β RII KO mice (KO) compared with control mice (Cntl) seven days after induction of pancreatitis. **B**. Immunostaining showed lower expression of p16 in acinar cells of TGF-β RII KO mice seven days after induction of pancreatitis. Arrows in the lower panels indicate interstitial cells that express similar p16 levels in the two strains. **C**. TUNEL staining (upper panel) and quantification (lower panel) showing comparable apoptosis of acinar cells in the two strains at the indicated time after induction of pancreatitis. Results are average ± SEM (n≥5), *p<0.05. Scale bars: 50 μM.

**Figure 4**. ADM is enhanced in the absence of TGF-β RII. **A**. Quantification of mice with ADM at the indicated times of pancreatitis induction showed higher incidence of ADM in TGF-β RII KO mice (KO) compared with control mice (Cntl). Data are expressed as percentage of the total number of mice (n>5). **B**. Left panel. Hematoxylin-Eosin (H&E)-stained sections of pancreata three days after cerulein treatment showed extended regions of ADM (asterisks) in KO mice. Right panel. Quantification of ADM area following cerulein-induced pancreatitis. Data are normalized by the total pancreatic area. **C**. Immunohistochemical analyses of KO mice after three days of pancreatitis revealed that, compared with the surrounding intact acinar tissue, ADM areas (asterisks) were negative for the acinar cell marker amylase, had a higher level of apoptotic cells (TUNEL) and similar number of replicating cells (Ki67). **D**. Immunostaining of phosphorylated EGFR (p-EGFR) showed increased labeling in ADM areas after the development of pancreatitis. Nuclei are stained with DAPI (blue). Results are average ± SEM (n≥5). Scale bars: 50 μM.

**Figure 5**. **A**. Immunostaining (left panel) and immunoblotting (right panel) of α-SMA showed higher activation of stellate cells in TGF-β RII KO

mice (KO) compared with control mice (Cntl). 40 µg proteins were processed for Western blotting and band intensity was normalized using GAPDH as a loading control. Results are average ± SEM (n=3). **B**. Similarly, quantification of Sirius red staining, expressed as percentage of total field area, showed higher collagen deposition in KO mice. **C**. Immunostaining and quantification of integrin $\alpha_v\beta_6$, expressed as percentage of total field area. Right panels, representative integrin $\alpha_V\beta_6$ immunostaining three days after induction of pancreatitis. Results are average ± SEM (n≥5). Scale bars: 50 µM.

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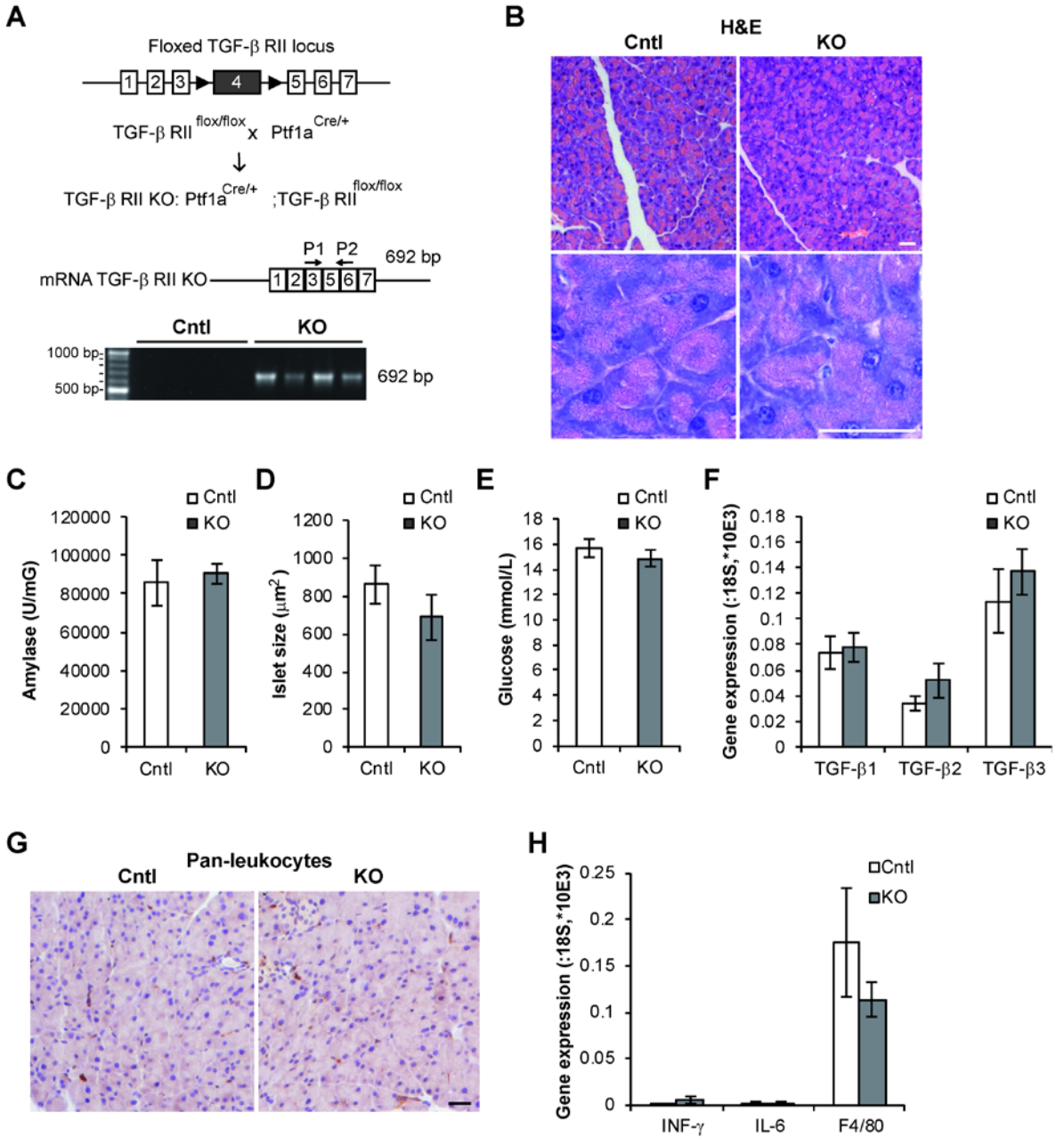
A
Floxed TGF-β RII locus
1 2 3 4 5 6 7
TGF-β RII flox/flox x Ptf1a Cre/+
TGF-β RII KO: Ptf1a Cre/+ ;TGF-β RII flox/flox
P1 P2
692 bp
mRNA TGF-β RII KO
1 2 3 5 6 7
Cntl
KO
1000 bp
500 bp
692 bp
B
H&E
Cntl
KO
C
Cntl
KO
Amylase (U/mG)
120000
100000
80000
60000
40000
20000
0
Cntl
KO
D
Cntl
KO
Islet size (μm2)
1200
1000
800
600
400
200
0
Cntl
KO
E
Cntl
KO
Glucose (mmol/L)
18
16
14
12
10
8
6
4
2
0
Cntl
KO
F
Cntl
KO
Gene expression (:18S,*10E3)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
TGF-β1
TGF-β2
TGF-β3
G
Pan-leukocytes
Cntl
KO
H
Cntl
KO
Gene expression (:18S,*10E3)
0.25
0.2
0.15
0.1
0.05
0
INF-γ
IL-6
F4/80

**Fig. 1**

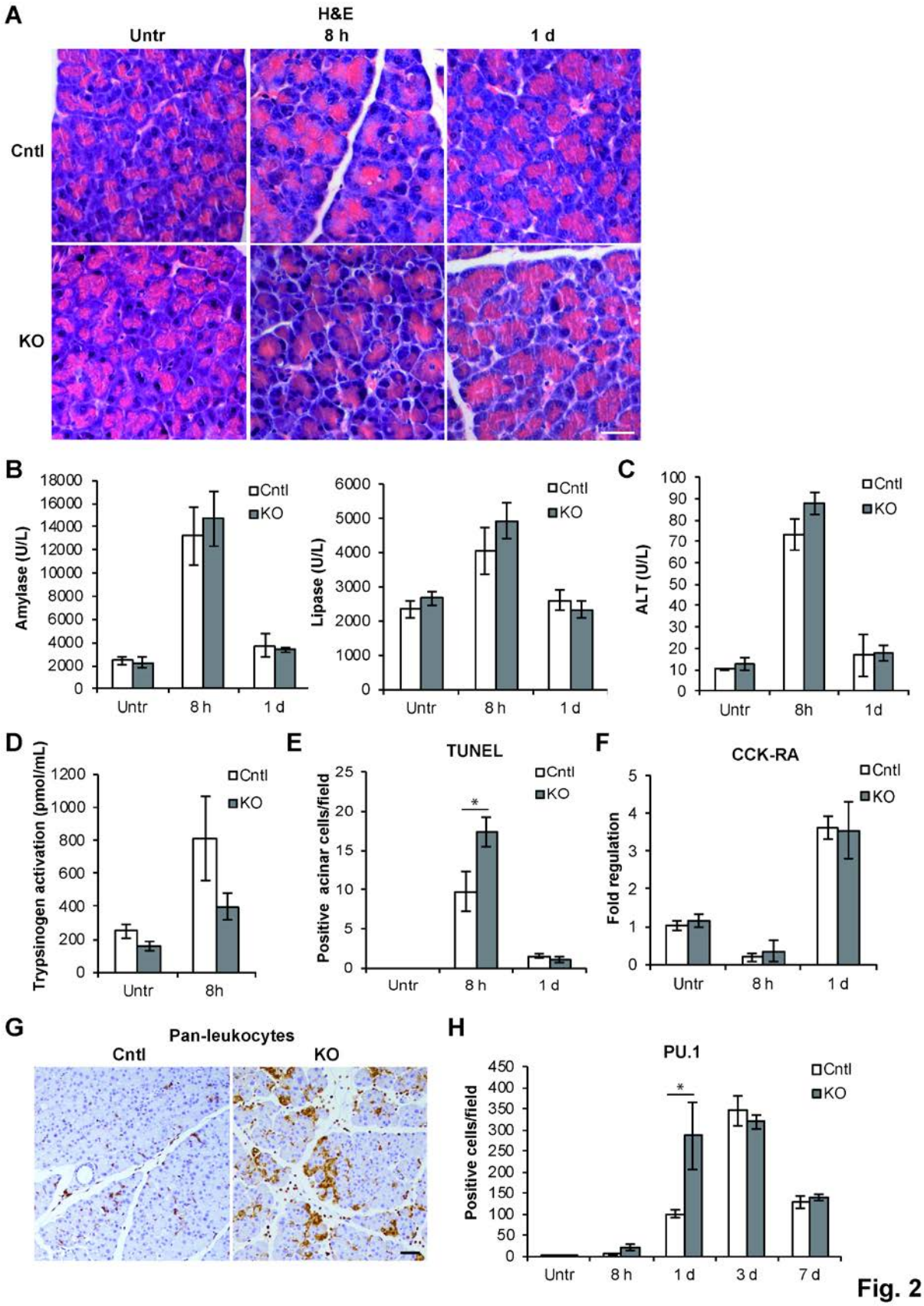
A
H&E
Untr
8 h
1 d
Cntl
KO
B
Amylase (U/L)
Lipase (U/L)
Cntl
KO
Untr
8 h
1 d
C
ALT (U/L)
8h
D
Trypsinogen activation (pmol/mL)
E
TUNEL
Positive acinar cells/field
F
CCK-RA
Fold regulation
G
Pan-leukocytes
H
PU.1
Positive cells/field
3 d
7 d

Fig. 2

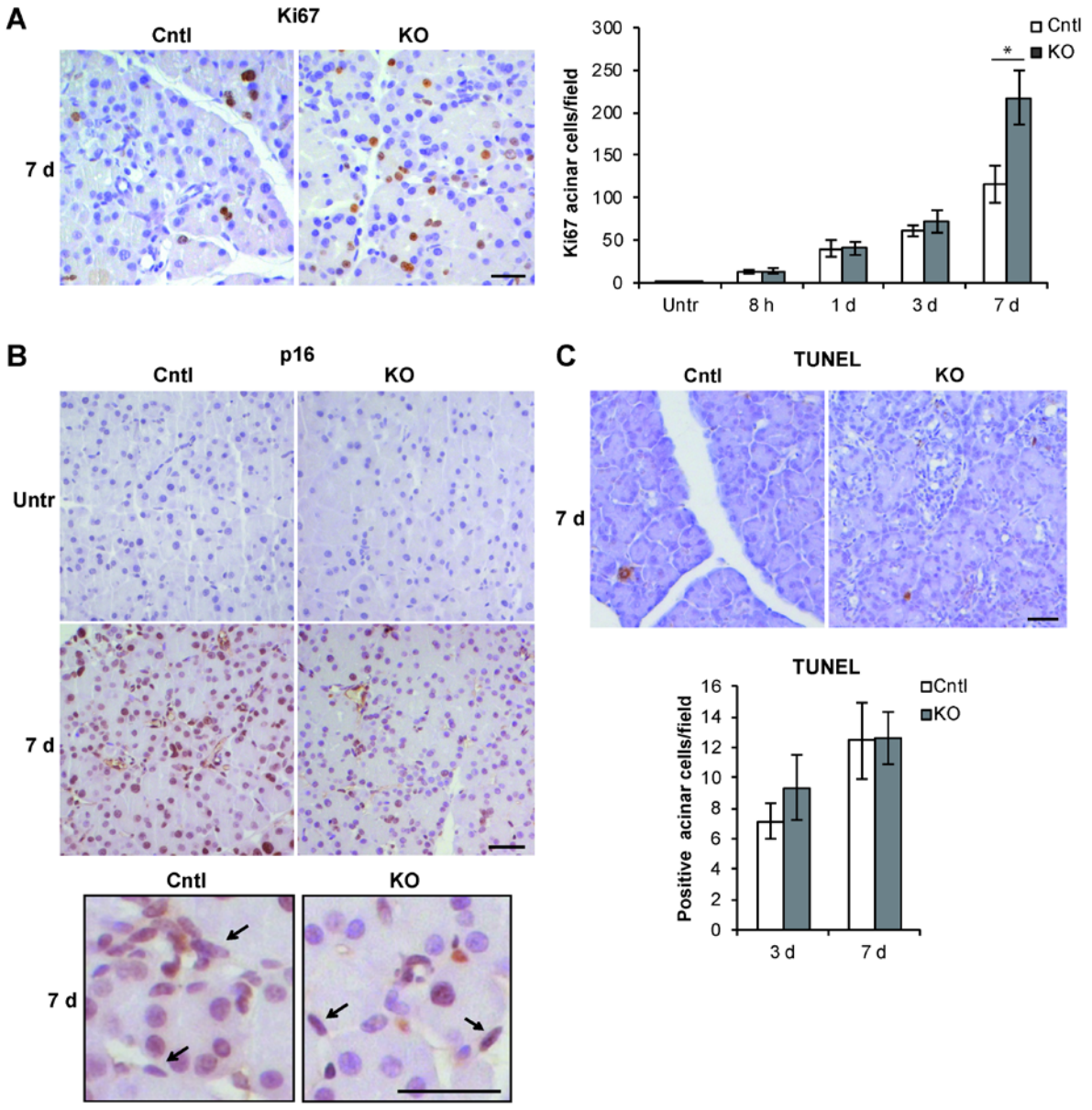
A
Ki67
Cntl
KO
7 d
Ki67 acinar cells/field
300
250
200
150
100
50
0
Cntl
KO
*
Untr
8 h
1 d
3 d
7 d
B
p16
Cntl
KO
Untr
7 d
Cntl
KO
7 d
C
TUNEL
Cntl
KO
7 d
TUNEL
Positive acinar cells/field
16
14
12
10
8
6
4
2
0
Cntl
KO
3 d
7 d

**Fig. 3**

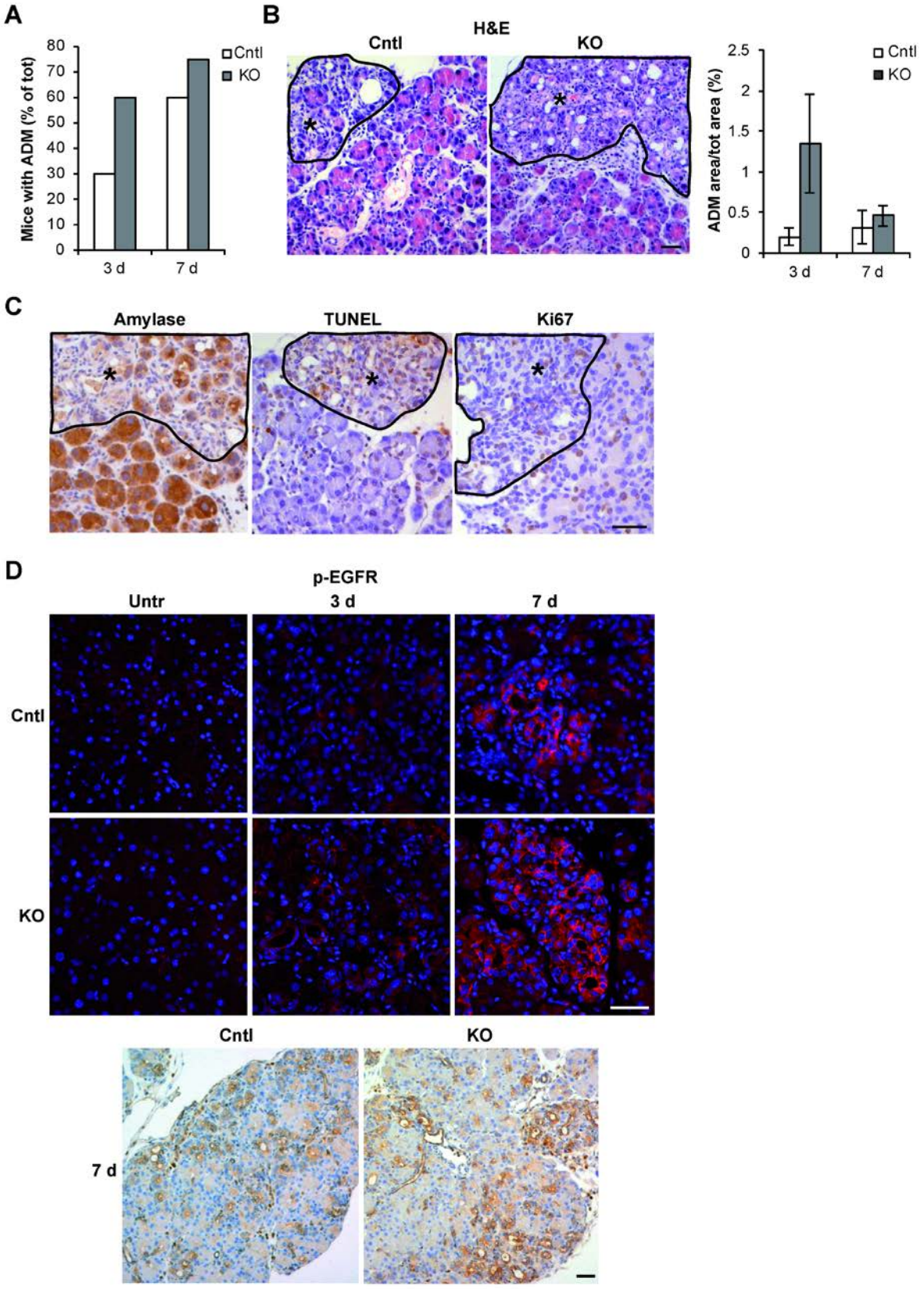
A
Mice with ADM (% of tot)
80
70
60
50
40
30
20
10
0
Cntl
KO
3 d
7 d
B
H&E
Cntl
KO
ADM area/tot area (%)
2.5
2
1.5
1
0.5
0
Cntl
KO
3 d
7 d
C
Amylase
TUNEL
Ki67
D
p-EGFR
Untr
3 d
7 d
Cntl
KO
Cntl
KO
7 d

Fig. 4

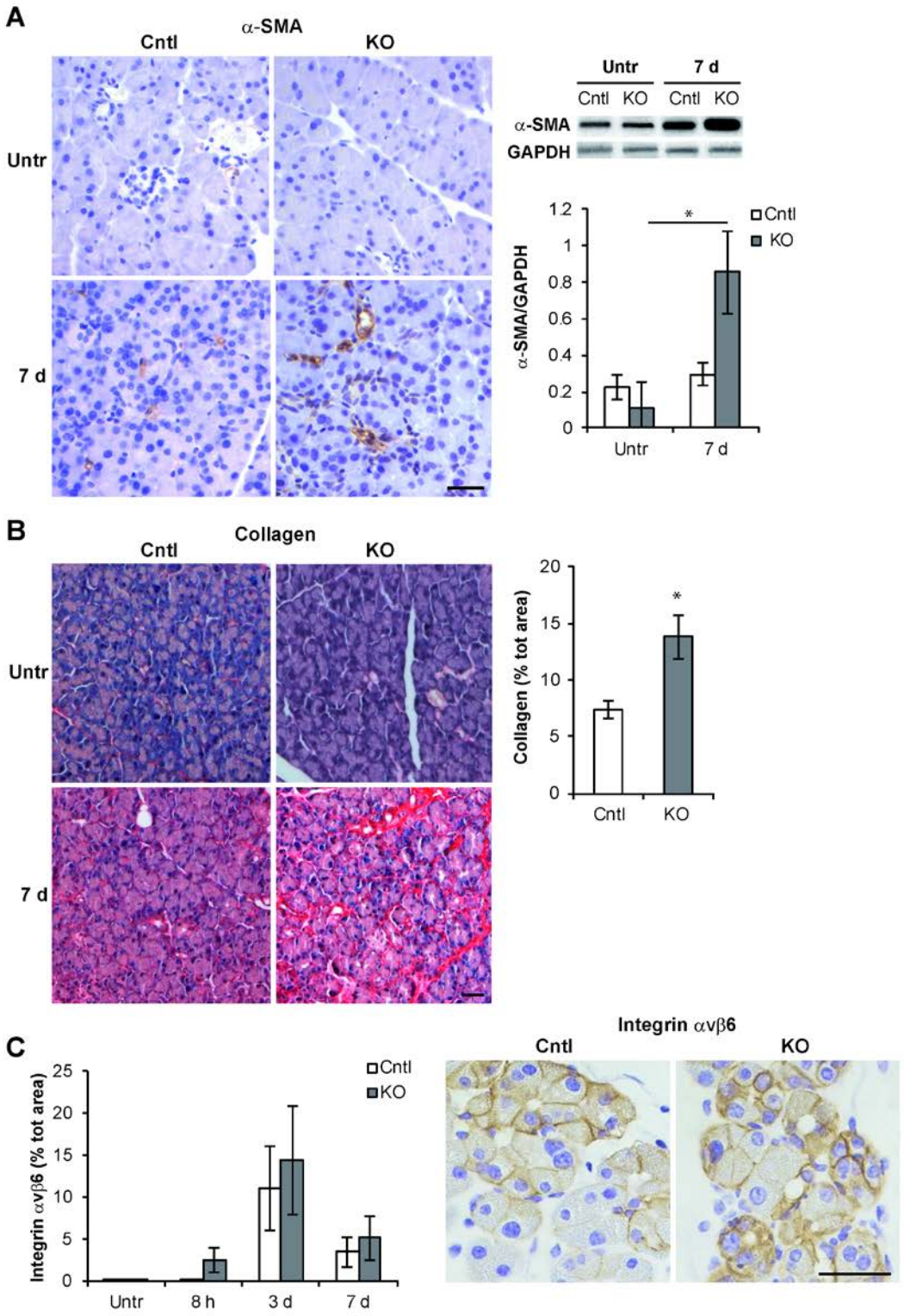
A
α-SMA
Cntl
KO
Untr
7 d
Untr
7 d
Cntl KO Cntl KO
α-SMA
GAPDH
α-SMA/GAPDH
Cntl
KO
Untr
7 d
B
Collagen
Cntl
KO
Untr
7 d
Collagen (% tot area)
Cntl
KO
C
Integrin αvβ6
Cntl
KO
Integrin αvβ6 (% tot area)
Cntl
KO
Untr
8 h
3 d
7 d

**Fig. 5**

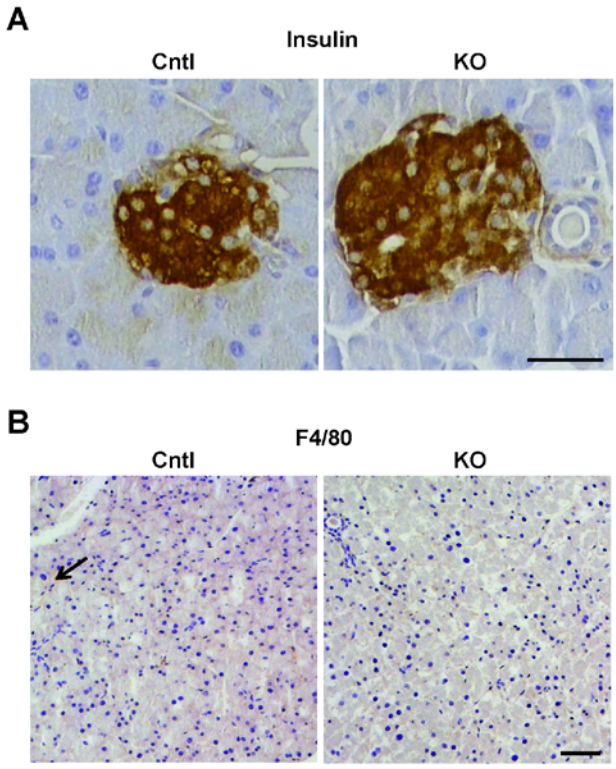


**Fig. S1**

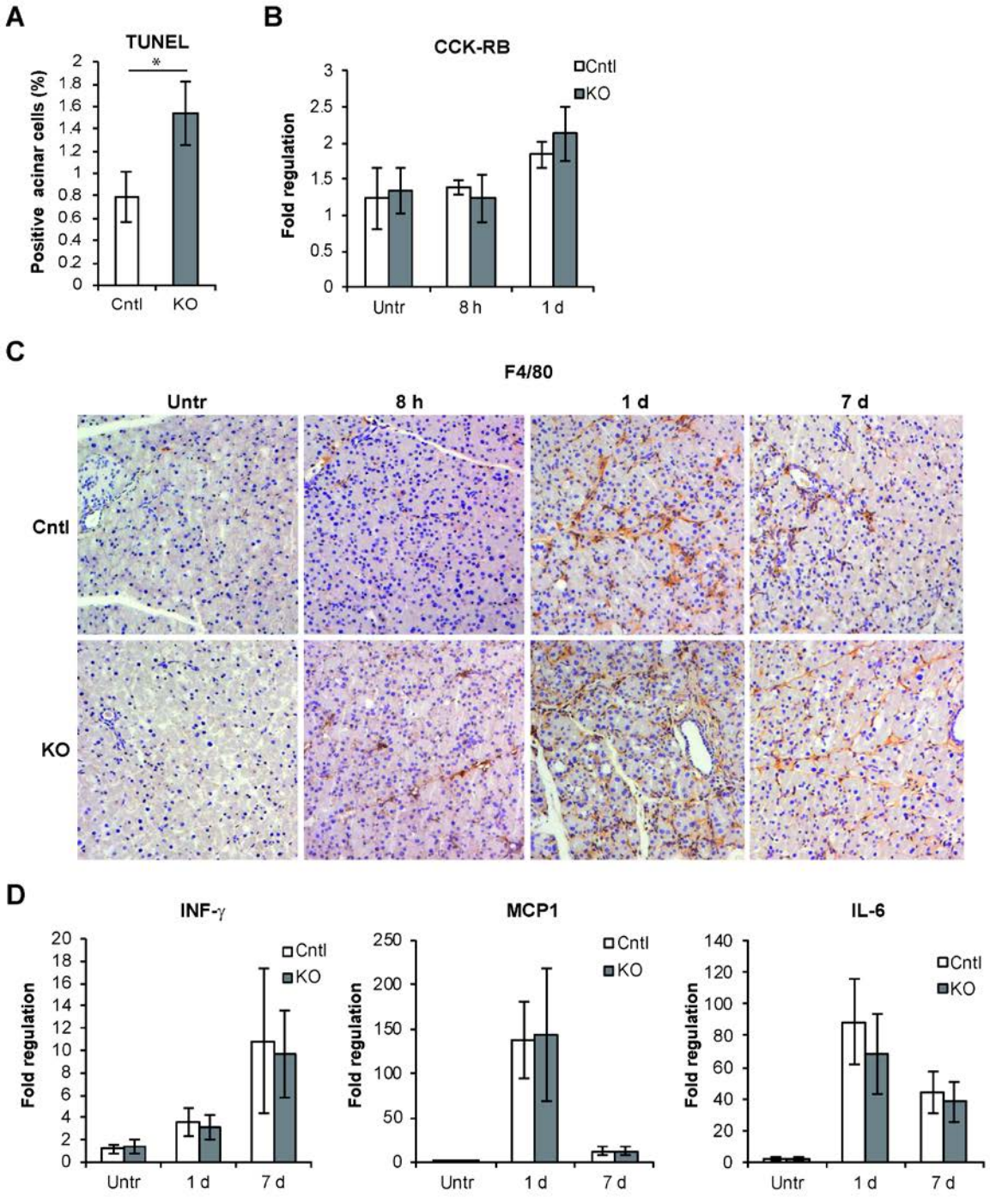
A
TUNEL
*
Positive acinar cells (%)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cntl
KO
B
CCK-RB
Cntl
KO
Fold regulation
3
2.5
2
1.5
1
0.5
0
Untr
8 h
1 d
C
F4/80
Untr
8 h
1 d
7 d
Cntl
KO
D
INF-γ
MCP1
IL-6
Cntl
KO
Fold regulation
20
18
16
14
12
10
8
6
4
2
0
250
200
150
100
50
140
120
100
80
60
40
20
Untr
1 d
7 d

Fig. S2

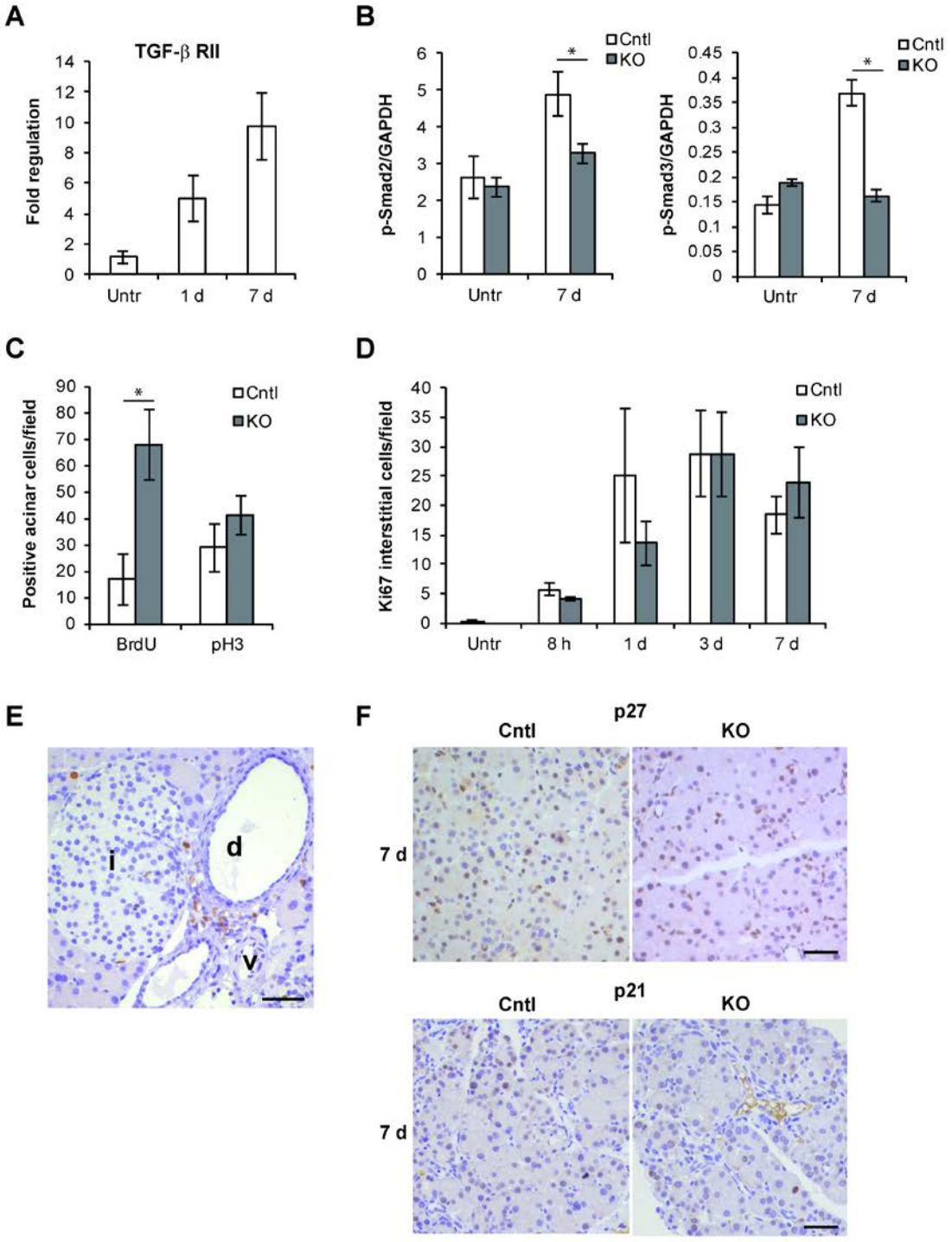
A
TGF-β RII
Fold regulation
Untr
1 d
7 d
B
p-Smad2/GAPDH
p-Smad3/GAPDH
Cntl
KO
Untr
7 d
C
Positive acinar cells/field
BrdU
pH3
D
Ki67 interstitial cells/field
Untr
8 h
1 d
3 d
7 d
E
i
d
v
F
p27
p21
Cntl
KO
7 d

**Fig. S3**

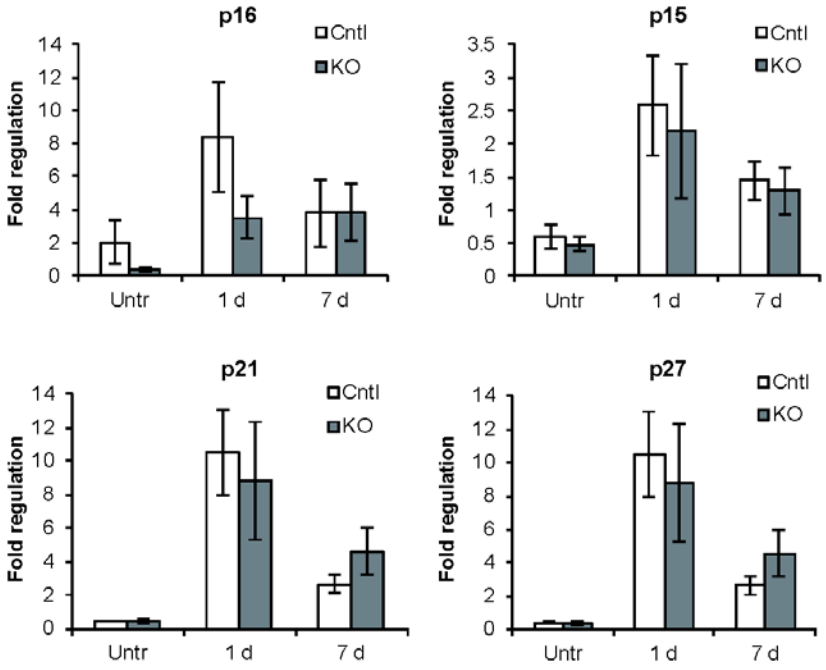
p16
Cntl
KO
Fold regulation
14
12
10
8
6
4
2
0
Untr
1 d
7 d
p15
Cntl
KO
Fold regulation
3.5
3
2.5
2
1.5
1
0.5
0
Untr
1 d
7 d
p21
Cntl
KO
Fold regulation
14
12
10
8
6
4
2
0
Untr
1 d
7 d
p27
Cntl
KO
Fold regulation
14
12
10
8
6
4
2
0
Untr
1 d
7 d

**Fig. S4**

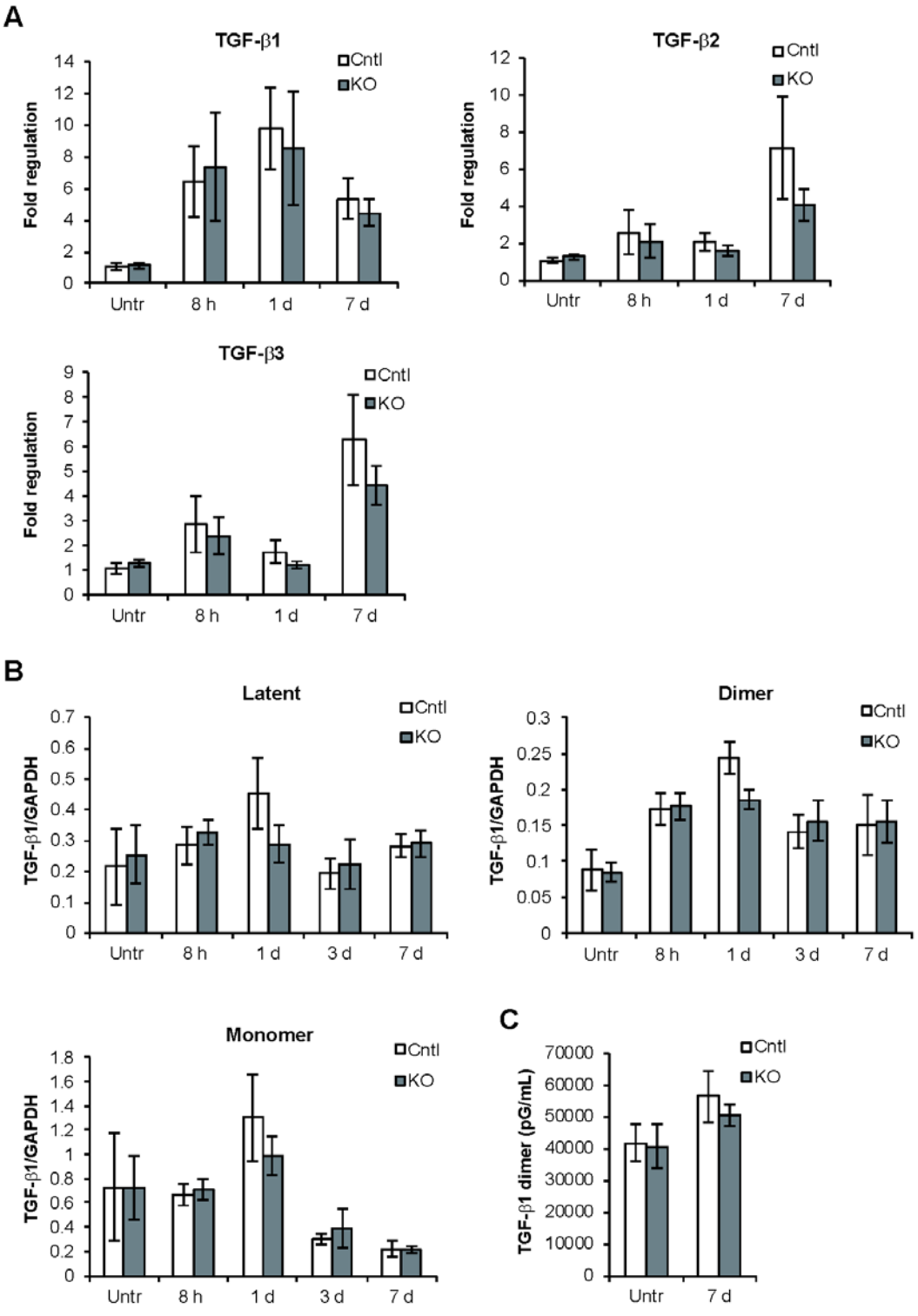
A
TGF-β1
Cntl
KO
Fold regulation
Untr
8 h
1 d
7 d
TGF-β2
TGF-β3
B
Latent
TGF-β1/GAPDH
3 d
Dimer
Monomer
C
TGF-β1 dimer (pG/mL)

**Fig. S5**

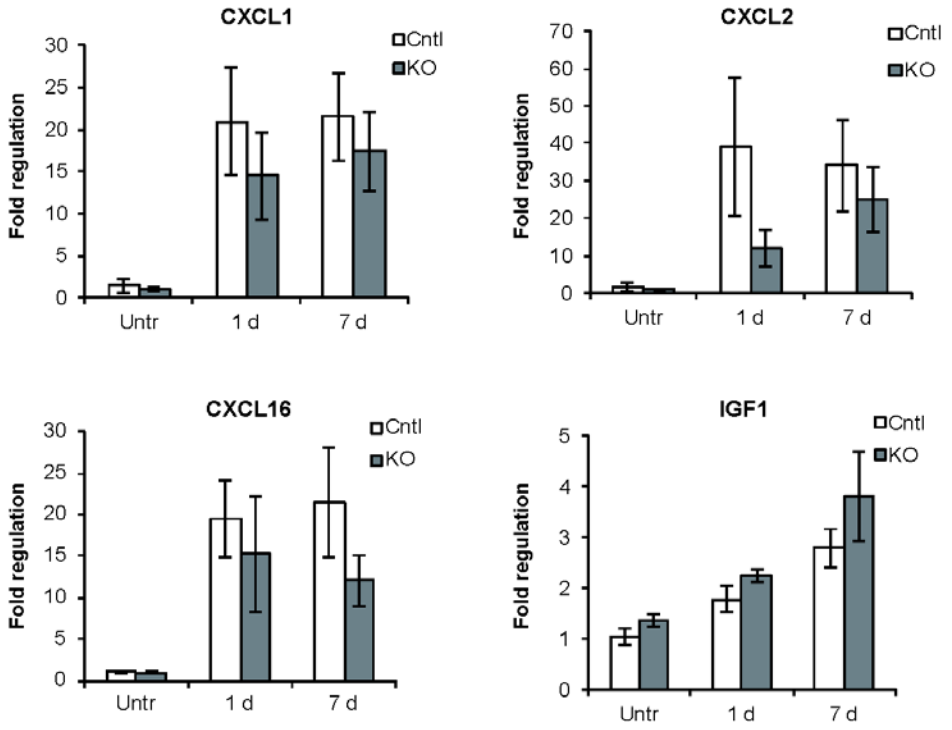
CXCL1
Cntl
KO
Fold regulation
30
25
20
15
10
5
0
Untr
1 d
7 d
CXCL2
Cntl
KO
Fold regulation
70
60
50
40
30
20
10
0
Untr
1 d
7 d
CXCL16
Cntl
KO
Fold regulation
30
25
20
15
10
5
0
Untr
1 d
7 d
IGF1
Cntl
KO
Fold regulation
5
4
3
2
1
0
Untr
1 d
7 d

**Fig. S6**

**Supplemental online material**
**Materials and Methods**

***Pancreatitis induction***

Animals received six hourly injections of 50 μg/kg cerulein on alternate days. Animals were treated on day 0, day 2 and day 4 and were harvested on day one after one set of injections, on day 3 after two sets of injections or on day 7 after three sets of injections, without cerulein treatment on the same day.

***Biochemical analysis of enzyme activity***

For determination of pancreatic enzymes present in the serum, blood was sampled by heart puncture. For determination of amylase levels present in pancreatic tissue, pancreata were homogenized with RIPA buffer containing protease inhibitor cocktail (complete ultra-tablets mini, Roche Diagnostics, Mannheim, Germany). Amylase and lipase levels were measured in the serum using the FUJIFILM DRI-CHEM 4000i analyzer (FIJIFILM Corporation, Tokyo, Japan).

For trypsinogen activation, 20 mG frozen pancreatic tissue was homogenized in 250 μL cold MOPS buffer (250 mM sucrose, 5 mM MOPS, 1 mM Mg SO4, pH 6.5) using the Precellys® 24 tissue homogenizer. Trypsin activity was measured flourometrically at 37°C in 250 μL trypsin assay buffer (50 mM Tris, 150 mM NaCl, 1 mM CaCl2, 0.1% BSA, pH 8.0) containing 250 μg supernatants and 40μM Boc-Glu-Ala-Arg-MCA (Bachem, Bubendorf, Switzerland) as a substrate, as described [1]. The fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored over a 10 min period.

***Immunohistochemistry***

Pancreas specimens were embedded in paraffin for histological analyses as described [2]. Primary antibodies used in this study were: anti-phospho-stat3 (Tyr705) (Cell signaling, Massachusetts, USA), rabbit anti-TGFβ1 (Santa Cruz Biotechnology inc, Santa Cruz, USA), rabbit anti-TGFβ2 (Santa Cruz Biotechnology inc, Santa Cruz, USA), rabbit anti-TGFβ3 (Abcam, Cambridge, UK), rabbit anti-phospho-smad2 (Ser465/467) (Millipore, Massachusetts, USA), insulin, rat anti-F4/80 (BMA Biomedicals, Augst, Switzerland), mouse anti-integrin αvβ6 (gift from Biogen Idec, Inc), rabbit anti-Pu.1 (Cell signaling, Massachusetts, USA), rabbit anti-phospho-histone 3 (Millipore, Massachusetts, USA), mouse anti-p21 (BD Pharmingen, NY, USA), rabbit anti-p27 (Santa Cruz Biotechnology inc, Santa Cruz, USA), mouse anti-αSMA (Dako, Glostrup, Denmark), rabbit anti-amylase (Sigma-Aldrich, Buchs, Switzerland), rabbit anti-coronin-1 (gift from Jean Pieters), rabbit anti-Ki67 (Abcam, Cambridge, UK), rabbit anti-p16 INK4B (Novus Biologicals, CO, USA), rabbit anti-Smad2/3 (Novus Biologicals, CO, USA), rabbit anti-phospho-Smad3 (Ser423/425) (Cell signaling, Massachusetts, USA), rabbit anti-phospho-EGFR (Millipore, Massachusetts, USA), rabbit anti-GAPDH (Sigma-Aldrich, Buchs, Switzerland). Secondary antibodies used in this study were: Biotinylated Goat Anti-Rabbit IgG (H+L), Biotinylated Goat Anti-Mouse IgG (H+L) and Biotinylated Goat Anti-Rat IgG (H+L). These antibodies are included in the VECTASTAIN® ABC kits. AlexaFluor 594 Goat Anti-Rabbit IgG, (Life Technologies, Carlsbad, California, USA).

Detection of DNA fragmentation in apoptotic cells was performed with a TUNEL assay using an ApopTag peroxidase Kit (Millipore, Massachusetts, USA). Collagen fibril deposition was detected with Sirius red staining. Microscopy analyses were performed on a Nikon Eclipse Ti fluorescence microscope (Amsterdam, The Netherlands).

***Western blotting***

Immunoblotting was performed by homogenizing tissue samples in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using a Bradford protein assay (BioRad, Hercules, CA, USA). Aliquots corresponding to 50 μg of proteins were separated by SDS-PAGE electrophoresis and blotting using a V3 Western Workflow system (BioRad, Hercules, CA, USA) according to manufacturer protocols. PVDF membranes were incubated with primary antibodies overnight at $4^{o}$C. All results were measured by densitometry and expressed by relative expression to GAPDH as a reference protein.

***Active TGFβ measurement***

Active TGF-β levels were quantified in blood serum using DuoSet ELISA kits (R&D Systems), in accordance with the manufacturer's instructions.

***Genomic DNA extraction and PCR***

Genomic DNA was extracted from pancreas tissue using a lysis buffer containing 100 mM Tris-HCl pH=8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg proteinase K/ml. The tissue was incubated over night at 55 ºC with agitation followed by 15 min proteinase K inactivation at 95 ºC. Mice were genotyped using conventional PCR according to manufacturer's instructions (Taq PCR Core Kit, Qiagen) with the following primer pairs: P1: 5'-GAAGGAAAAGAAAAGGG-3', P2: 5'-CCAGCACTCGGTCAAAG-3'; P5: 5-TATTGGGTGTGGTTGTGGACTTTA-3, P3: 5-TATGGACTGGCTGCTTTTGTATTC-3. P3 and P5 primer pair was used to screen for mice

containing the null alleles and yielding positive 692bp PCR product. P1 and P2 primer pair was used to screen for TGF-βRII encoding sequence between exon 4 and exon 8. The samples where exon 4 was intact yielded a 1195bp PCR product.

***Transcript analysis***

The following Taqman probes (Applied Biosystems) were used: MCP1 (CCL2) Mm00441242_m1, IL-6 Mm00446190_m1, IL-1β Mm00434228_m1, p21 (WAF1) Mm00432448_m1, p27 (Cdkn1b) Mm00438168_m1, p15 (Cdkn2b) Mm00483241_m1, p16 (Cdkn2a) Mm00494449_m1, TGF-β1 Mm00441724_m1, TGF-β2 Mm00436955_m1, TGF-β3 Mm00436960_m1, EGFR Mm00433023_m1, EGF Mm00438696_m1, IGF1 Mm00439560_m1, TGF-βRII Mm00436977_m1, F4/80 Mm00802530_m1, INF-γ Mm01168134_m1, CCKAR Mm00438060_m1, CCKBR Mm00432329_m1, CXCL1 Mm04207460_m1, CXCL2 Mm00436450_m1, CXCL16 Mm00469712_m1.

***Statistical analyses***

Groups of 5 animals were tested for each experiment. The data are expressed as means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test or one-way analysis of variance (GraphPad Prism 4.0c; GraphPad Software, Inc.), and a probability value <0.05 was considered statistically significant. When the overall probability value was <0.05, the Dunnett multiple-comparisons test was used as a post-test to determine whether there was a significant difference between values of control (reference sample) and samples of interest.

**Figure legends**

**Figure S1**. **A**. Immunostaining of insulin in untreated control (Cntl) and TGF-β RII KO (KO) mice. B. Immunostaining of macrophage marker F4/80 in untreated pancreatic tissue. Scale bars: 50 μM.

**Figure S2**. **A**. Quantification of TUNEL positive acinar cells expressed as percentage of total acinar cell number in control (Cntl) and TGF-β RII KO (KO) mice eight hours after induction of pancreatitis. **B**. qPCR of CCK-B receptor in Cntl and KO pancreata at the indicated time following induction of pancreatitis. **C**. Immunostaining of macrophages (F4/80) after pancreatitis induction. **D**. qPCR of interferon-γINF-γ), monocyte chemoattractant protein 1 (MCP1) and interleukin 6 (IL-6) in Cntl and KO pancreata at the indicated time following induction of pancreatitis. Results are average ± SEM (n≥5), *p<0.05.

**Figure S3**. **A**. qPCR of TGF-β RII in control mice at the indicated time following induction of pancreatitis. **B**. Immunoblotting of 40 μg proteins showed up-regulation of phosphorylated Smad3 and Smad3 in control (Cntl) but not in TGF-β RII KO (KO) pancreata following pancreatitis induction. Band intensity was normalized using GAPDH as a loading control. **C**. Quantification of BrdU and phopsho-histone 3 (pH3) positive pancreatic acinar cells in Cntl and KO mice seven days after induction of pancreatitis. **D**. Quantification of Ki67 positive interstitial cells at the indicated time following induction of pancreatitis. **E**. Immunostaining of Ki67 did not reveal increased replication of islet (i), ductal (d) and vascular (v) cells in KO mice following pancreatitis induction. Three days of treatment are shown. **F**. Immunostaining showed comparable expression of p27 and p21 in acinar cells of TGF-β RII KO mice seven days after induction of pancreatitis. Results are average ± SEM (n≥5), *p<0.05. Scale bars: 50 μM.

**Figure S4**. qPCR of cyclin-dependent kinase inhibitors in control (Cntl) and TGF-β RII KO (KO) pancreata at the indicated time following induction of pancreatitis. Results are average ± SEM (n≥5).

**Figure S5**. **A**. qPCR of TGF-β isoforms in control (Cntl) and TGF-β RII KO (KO) pancreata at the indicated time following induction of pancreatitis. **B**. Immunoblotting of TGF-β1latent, active dimer and monomer forms in Cntl and KO pancreata at the indicated time following induction of pancreatitis. Band intensity was normalized using GAPDH as a loading control. **C**. Quantification of active TGF-β1 dimer in Cntl and KO blood following induction of pancreatitis. Results are average ± SEM (n≥5).

**Figure S6**. qPCR of pro-fibrotic cytokines in control (Cntl) and TGF-β RII KO (KO) pancreata at the indicated time following induction of pancreatitis. Results are average ± SEM (n≥5).

1. Kawabata S, Miura T, Morita T*, et al.* Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood-clotting proteases and trypsin. *European journal of biochemistry / FEBS* 1988; **172**: 17-25.

2. Silva A, Weber A, Bain M*, et al.* COX-2 is not required for the development of murine chronic pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G968-975.

# 6. Manuscript C

## Serotonin promotes acinar dedifferentiation following pancreatitis-induced regeneration in the adult pancreas

**Enrica Saponara[1], Kamile Grabliauskaite[1], Marta Bombardo[1], Alberto B Silva[1], Yinghua Tian[1], Elisabeth M Schraner[2], Theresia Reding[1], Sabrina Sonda[1] and Rolf Graf[1]**

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Submitted at *Journal of Pathology* 2015

**Contribution:**

I contributed to this study by characterizing the replication of acinar cells in the absence of serotonin and by revising the manuscript.

**Serotonin promotes acinar dedifferentiation following pancreatitis-induced regeneration in the adult pancreas.**

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**Running title:** serotonin and acinar cell de-differentiation

# *Contributed equally*

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**Competing interests:** none

**Word count:** 3477

## Abstract

The exocrine pancreas exhibits a distinctive capacity for tissue regeneration and renewal following injury. This regenerative ability has important implications for a variety of disorders, including pancreatitis and pancreatic cancer, diseases associated with high morbidity and mortality. Thus, understanding its underlying mechanisms may help develop therapeutic interventions. Serotonin has been recognized as a potent mitogen for a variety of cells and tissues. Here we investigated whether serotonin exerts a mitogenic effect in pancreatic acinar cells in three regenerative models, namely inflammatory tissue injury following pancreatitis, tissue loss following partial pancreatectomy and thyroid hormone stimulated acinar proliferation. Genetic and pharmacological techniques were used to modulate serotonin levels *in vivo*. Acinar de-differentiation and cell cycle progression during the regenerative phase were investigated over the course of two weeks. By comparing acinar proliferation in the different murine models of regeneration, we found that serotonin did not affect clonal regeneration of mature acinar cells. However, it was required for acinar de-differentiation following inflammation-mediated tissue injury. Specifically, lack of serotonin resulted in delayed up-regulation of progenitor genes, delayed formation of acinar-to-ductal metaplasia and defective acinar cell proliferation. We identified serotonin-dependent acinar secretion as a key step in the progenitor-based regeneration, as it promoted acinar cell de-differentiation and the recruitment of type 2 macrophages. Finally, we identified a regulatory Hes1-Ptfa axis in the uninjured adult pancreas activated by zymogen secretion. Our findings indicate that serotonin plays a critical role in the regeneration of the adult pancreas following pancreatitis by promoting de-differentiation of acinar cells.

**Keywords:** serotonin; acinar secretion; de-differentiation; regeneration; ADM; pancreatitis; partial pancreatectomy.

## Introduction

Despite a low homeostatic turnover, the adult exocrine pancreas has the ability to regenerate following an inflammatory insult. An increasing number of reports have revealed that during this regenerative process acinar cells, which constitute the most abundant cell population of the exocrine tissue, transiently de-differentiate and initiate a genetic program resembling the one found in embryonic pancreatic precursors (recently reviewed in [1,2]). Although numerous studies have identified crucial factors involved in the regeneration of acinar cells, the precise mechanisms initiating the cascade of events

leading to their de-differentiation have not yet been identified. Serotonin (5-hydroxytryptamine, 5-HT), a monoamine stored and released by circulating platelets, has long been recognized as a potent bioactive molecule with mitogenic effects not only during embryonic development [3,4] but also in adult cells [5]. Examples of the mitogenic role of 5-HT in adult tissues include liver regeneration following hepatectomy [6], megakaryocyte colony formation [7], pulmonary artery smooth muscle cell proliferation [8] and beta cell expansion during pregnancy [9].

We recently showed that 5-HT plays a major role in the pathophysiology of the exocrine pancreas where it promotes acinar cell secretion and exacerbates the severity of inflammation at the onset of acute pancreatitis [10]. Here we investigated whether 5-HT acts as a mitogen for pancreatic acinar cells and thus promotes tissue regeneration. To this aim, we compared three types of regenerative stimuli, namely cerulein-induced pancreatitis, partial pancreatectomy and thyroid hormone supplementation, in mice deficient in peripheral 5-HT (tryptophan hydroxylase 1 knocked-out, TPH1$^{-/-}$), and in wild type mice supplemented with 5-HT precursor.

## Material and methods

### *Animal experiments*

All animal experiments were performed in accordance with Swiss federal animal regulations and approved by the cantonal veterinary office of Zurich. Pancreatitis was induced in adult (8-10 weeks of age) wild-type (WT) C57BL/6 (Harlan Laboratories, Horst, The Nederlands) and TPH1$^{-/-}$ mice [11] on a C57BL/6 background (own breeding) via six intra peritoneal (i.p.) injections of 50 μg/kg cerulein administered hourly over a two week period, as described in Supplementary Materials and Methods and [12]. Pancreatectomy was performed according to [13], with the procedure adapted to remove 60% of the organ. 400 μg/kg 3,5,3-L-tri-iodothyronine (T3) was administered daily via i.p. injections for six days. 20 mg/kg 5-hydroxytryptophan (5-HTP) was administered by two subcutaneous injections per day, 12 hours apart. The treatment was started one day before cerulein treatment; control animals received 0.9% NaCl injections.

### *Histology, immunohistochemistry and immunoblotting*

Detailed protocols and primary antibodies used in this study are listed in Supplementary Materials and Methods. Quantification of labelled cells was performed in at least 10 randomly selected high-power fields (×100) per slide. Non-acinar tissue areas (islets, vessels, fibrotic tissue) were excluded from the analysis.

### *Electron microscopy analysis*

WT and TPH1$^{-/-}$ mice were harvested 8 hours after the beginning of cerulein treatment. Sliced pancreata were fixed in 2% glutaraldehyde/1% paraformaldehyde in 0.1M Na/K-phosphate for 90 min. After washing, the samples were postfixed with 1% osmium tetroxide in 0.1M Na/K-phosphate for one hour, dehydrated in a graded ethanol series, transferred to acetone for embedding in Epon and polymerized at 60°C for 2.5 days. Ultrathin sections were stained with uranyl acetate and lead citrate and examined at an acceleration voltage of 100 kV in a Philips CM 12 transmission electron microscope (Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000, Gatan, Pleasanton, CA).

### *Transcript analysis*

Total RNA was extracted from pancreata as previously described [14] and reverse transcribed with qScript™ cDNA SuperMix (Quanta Biosciences). Transcript levels were normalized using 18S rRNA as a reference and expressed as fold regulation relative to the value of untreated control animals. Taqman probes (Applied Biosystems) used in this study are listed in Supplementary Materials and Methods.

## Results

### Lack of 5-HT results in defective acinar cell proliferation and delayed ADM formation

To test whether 5-HT is mitogenic for acinar cells, we compared acinar proliferation following induction of pancreatitis in WT and in TPH1$^{-/-}$ mice, characterized by reduced 5-HT levels in the periphery but normal 5-HT signaling in the nervous system [10]. Unexpectedly, TPH1$^{-/-}$ animals showed a significantly higher number of acinar cells positive for the general cell cycle activation marker Ki67 after one week of cerulein treatment compared with WT animals, while the number of bromodeoxyuridine (BrdU) positive cells in G1/S phase was similar in the two strains. Phospho-histone H3 (pH3) positive acinar cells in G2/M phase increased in TPH1$^{-/-}$ mice only 14 days after the beginning of the treatment (Fig. 1A, S1A). Acinar and interstitial cells were distinguished based on nuclear size, shape and location, as shown in Fig. S1B, C. Quantification of of acinar cells in G2 phase, displaying punctuate pH3 staining, and in mitotic phase, with homogeneous nuclear pH3 staining [15], showed a reduced transition into mitotic phase in TPH1$^{-/-}$ animals one week after induction of pancreatitis (Fig. 1B).

In addition, formation of acinar-to-ductal metaplasia (ADM), a transient trans-differentiation observed during pancreatitis-induced regeneration, was delayed in TPH1$^{-/-}$ mice three days after induction of pancreatitis (Fig. 1C). The slower kinetics of ADM formation

was not the result of defective expression or re-localization of β-catenin, a key a transcriptional activator promoting ADM formation [16]. β-catenin was similarly localized on the plasma membrane and nuclei of intact acinar cells in both strains (Fig. 1D) and was also found in ADM of WT animals and in the rare areas with reduced amylase content in TPH1$^{-/-}$ mice (Fig. 1E, arrowheads). Seven days after pancreatitis, mature clustered ductal structures were visible in TPH1$^{-/-}$ mice and were similar to WT ADMs in terms of β-catenin expression, Sox9 up-regulation, amylase down-regulation and activation of αSma-positive stellate cells (Fig. 2A, S2). Taken together, these data indicate that, upon cerulein stimulation, TPH1$^{-/-}$ acinar cells are able to initiate the cell cycle; however, they display defective cell cycle progression and delayed ADM formation.

**Lack of 5-HT delays acinar de-differentiation during pancreatitis**

Proliferation of pancreatic acinar cells is preceded by a temporary de-differentiation and up-regulation of markers normally associated with pancreatic ductal or progenitor cells [17]. Concomitant with reduced ADM formation, TPH1$^{-/-}$ mice showed delayed up-regulation of the ductal marker cytokeratin 19 (CK19) three days after induction of pancreatitis (Fig. 2A, B). In addition, delayed up-regulation was also observed for several progenitor markers, including Sox9, Hes1, Notch1, Hnf1β. In addition, ductal and progenitor gene expression was higher in TPH1$^{-/-}$ than in WT animals after 14 days of pancreatitis, with the exception of Aldh1, the expression of which was comparable in the two strains (Fig. 2A, B).

Noteworthy, delayed progenitor gene up-regulation in TPH1$^{-/-}$ mice was not accompanied by defective down-regulation of genes typical of the differentiated state, such as secretory granule enzymes and transcription factors controlling acinar cell identity, such as Mist-1[18-20] (Fig. S3). These data indicate that WT and TPH1$^{-/-}$ mice responded equally to the cerulein stimulus by down-regulating genes specific to mature differentiated acinar cells. However, amylase content did not decrease in TPH1$^{-/-}$ acini, as quantified by western blotting (Fig. 3A), enzymatic activity (Fig. 3B) and immunofluorescence analysis (Fig. 3C), indicating that down-regulation of zymogen transcripts was not sufficient to induce zymogen loss in these mice. Autophagy can reduce zymogen content by intracellular degradation [21]. However, autophagy was not differentially regulated in TPH1$^{-/-}$ mice, as shown by comparable processing of the autophagic protein LC3 (Fig. S4A). Similarly, autophagic marker p62 [22] showed widespread expression in acini at 8 hours and down-regulation one day after induction of pancreatitis in both strains (Fig. S4B, C).

**Increased zymogen secretion promotes acinar de-differentiation during acute pancreatitis**

An alternative cellular mechanism explaining zymogen loss observed in acinar cells following pancreatitis could be the secretion of zymogens into extracellular space. We previously showed that TPH1$^{-/-}$ mice have impaired zymogen secretion [10]. This defective secretory process was further investigated at the ultrastructural level. Electron microscopy analysis revealed that zymogen granules were more tightly packed in TPH1$^{-/-}$ than in WT mice, both in control and after cerulein treatment (Fig. 3D). Furthermore, size distribution analysis showed that granule size, comparable in the two strains in the untreated controls, increased after stimulation of secretion, indicative of granule swelling preceding content expulsion [23]; however, the enlargement was higher in TPH1$^{-/-}$ (Fig. 3E), presumably a consequence of impaired secretion. Thus, our results show that reduced zymogen secretion observed in the absence of 5-HT is associated with defective zymogen loss followed by delayed up-regulation of progenitor genes. To test whether 5-HT-dependent zymogen secretion is required to initiate acinar de-differentiation following pancreatitis, we increased zymogen secretion 24 hours before cerulein administration by treatment with 5-hydroxytryptophan (5-HTP) (regimen scheme depicted in Fig. 4A), a precursor of serotonin that dose-dependently stimulates apical secretion of zymogens into pancreatic ducts (reviewed in [24]). This stimulated secretion is associated with a protective effect during induction of acute pancreatitis [25-27]. Under 5-HTP supplementation and one day of pancreatitis, we also observed lower levels of inflammatory infiltrates in the pancreas (Fig. 4B), chemokine expression (Fig. 4C) and serum amylase (Fig. 4D), likely due to the increased 5-HTP-stimulated apical secretion counteracting the aberrant baso-lateral secretion induced by cerulein. The reduced severity of the disease did not translate in reduced acinar cell de-differentiation, as 5-HTP+cerulein-treated mice showed robust amylase loss in the pancreas similar to cerulein-treated mice (Fig. 4E). In addition, these mice had increased RNA levels of progenitor gene Hes-1 (Fig. 4F) and earlier expression of Sox-9 in acinar cell nuclei (Fig. 4G). These results suggest that stimulation of zymogen secretion enhances acinar de-differentiation following induction of pancreatitis. However, despite reducing the initial inflammation, 5-HTP supplementation (regimen scheme depicted in Fig. S5A) was not sufficient

to promote pancreatic regeneration, as shown by comparable levels of acinar and interstitial cell replication (Fig. S5B) and inflammatory cell infiltration (Fig. S5C) three days after pancreatitis induction. In addition, increasing the dosa of 5-HTP supplementation stimulated the expression of progenitor (Fig. S5D) and inflammatory genes (Fig. S5E) compared to one set of 5-HTP. Six doses of 5-HTP supplementation further boosted the up-regulation of inflammatory genes (Fig. S5F), and resulted in an increased trend of acinar cell replication (Fig. S5G), ADM formation (Fig. S5H) and inflammatory infiltration (Fig. S5I), suggesting that supplementation with exogenous 5-HTP did not ameliorate disease progression.

**Lack of 5-HT delays the expression of MMPs during pancreatitis**

As inflammatory cells play an active role in ADM formation, we then tested the level of pancreatic inflammation in the two strains. TPH1$^{-/-}$ mice showed reduced inflammation after one [10] and three days of pancreatitis (Fig. 4H, S6A). At this time point, expression of type-2 macrophage markers Mgl1 and Mrc1 was also reduced in TPH1$^{-/-}$ mice, while the type-1 marker Nos2 was comparable in the two strains (Fig. 4I), Macrophage marker expression increased in TPH1$^{-/-}$ mice at later time points (Fig. S6B). Cytokines secreted by macrophages stimulate matrix metalloproteinase (MMP) synthesis and secretion in acinar cells, thus driving digestion of extracellular matrix and ADM formation [28]. Concomitant with reduced macrophage infiltration, TPH1$^{-/-}$ mice showed a reduced trend of MMP7 (Fig. 4L) and TNF$\alpha$ expression (Fig. S6C). Analysis of vascular associated factors, including CD146 and VEGF, did not show alterations in the two strains after three days of pancreatitis, when the major ADM difference was observed, but their expression remained elevated in TPH1$^{-/-}$ mice after 14 days of the disease (Fig. S6D).

To test whether a reduced extracellular matrix digestion contributed to delayed ADM formation, we isolated pancreatic acini with collagenase and compared their de-differentiation after culture in suspension for 24 hours, as described [29]. In this *in vitro* setting, de-differentiation was comparable in WT and TPH1$^{-/-}$ acini, with or without exogenous 5-HT added to the medium (Fig. 4M), suggesting that the 5-HT mediated effect is exerted up-stream of extracellular matrix digestion.

**Lack of 5-HT does not alter clonal regeneration of acinar cells following partial pancreatectomy and T3 administration.**

As we found that 5-HT supports progenitor-based regeneration following pancreatitis, we then investigated whether zymogen secretion is also required for pancreatic regeneration that does not depend on robust acinar de-differentiation and activation of progenitor genes. To this aim, we analyzed acinar replication following 60% partial pancreatectomy (PPX) that triggers a regenerative response preferentially based on clonal division of differentiated acinar cells. Acinar replication, albeit lower than the one induced after pancreatitis at all the time points analyzed, peaked one week following PPX (Fig. 5A). Importantly, TPH1$^{-/-}$ mice did not show accumulation of Ki67 positive acinar cells (Fig. 5A, B). The replication peak was not preceded by robust expression of progenitor genes, the up-regulation of which was lower than during pancreatitis and comparable in the two strains, with the exception of Hes1 that increased at the two week time point only in WT mice (Fig. 5C). In addition, pancreata isolated after pancreatic resection showed comparable amylase content (Fig. 5D) and minimal levels of inflammation, apoptosis and fibrosis in the two strains (Fig.S7A-C). To test the effect of 5-HT in another model of inflammation-independent regeneration, we induced acinar proliferation by administering the thyroid hormone 3,5,3-L-tri-iodothyronine (T3) [30,31]. T3 treated WT and TPH1$^{-/-}$ mice showed similar levels of acinar replication (Fig. 5E) and minimal leukocyte infiltration (Fig. 5F). Collectively, these data revealed that acinar replication following PPX and T3 administration is not affected by the lack of 5-HT.

**5-HT receptors are up-regulated during pancreatic regeneration**

As 5-HT signals via specific 5-HT receptors and transporter, we then compared their expression during pancreatic regeneration. Type 1 and 2 receptors, known to play a role in regeneration of liver and heart [6,32,33] and found up-regulated in pancreatic cancer [34], were up-regulated following pancreatitis in WT mice and showed a delayed kinetic in TPH1$^{-/-}$ animals (Fig. S8A). Analysis of samples with reduced inflammation, such as TPH1$^{-/-}$ and 5-HTP supplemented WT mice one day after pancreatitis, indicated that inflammatory cells substantially contribute to the observed level of receptor expression, except in the case of 5-HT2A (Fig. S8B). Similarly, PPX samples, characterized by minimal tissue inflammation, showed lower 5-HT receptor and transporter expression compared with pancreatitis specimens (Fig. S8C). Interestingly, expression of 5-HT2B robustly increased in WT mice 14 days after PPX, when up-regulation of progenitor Hes1 was observed.

To test whether acinar cells also express 5-HT receptors and transporter, we isolated pancreatic acini one day after pancreatitis induction. Expression of 5-HT receptors but not transporter increased in acinar cells following the disease

and TPH1$^{-/-}$ acini had an opposite trend of receptor expression compared with WT cells, showing lower type 1 and higher type 2 receptor levels (Fig. S8D). Collectively, these results indicate that pancreatic regeneration is associated with up-regulation of 5-HT receptors and transporter, which can be expressed not only by infiltrating cells but also by pancreatic acini.

**Hes1-Ptf1a axis is activated in the uninjured adult pancreas**

As we observed that 5-HTP treatment enhanced acinar de-differentiation following induction of pancreatitis (Fig. 4E, F), we then tested whether this regulation was observed also in the absence of tissue damage. 5-HTP-treated WT mice had normal levels of serum and tissue amylase (Fig. 4C, D) and no Sox9 expression in acinar cells (Fig. 6A), suggesting that increased secretion into ducts is not sufficient to activate acinar de-differentiation. However, 5-HTP treatment increased the expression of the progenitor gene Hes1 in the pancreas (Fig. 6B), albeit at a lower level than following 5-HTP+cerulein treatment. Hes1 is a critical orchestrator of pancreatic embryogenesis (reviewed in [35]), as it regulates maintenance of progenitor cells and timing of cell differentiation by antagonizing the function of Ptf1a, a basic helix-loop-helix transcription factor that promotes zymogen synthesis [36]. Hes1 up-regulation following 5-HTP treatment was concomitant with Ptf1a down-regulation (Fig. 6B). Moreover, early time points following cerulein treatment showed initial Ptf1a up-regulation followed by Hes1 expression and subsequent Pft1a down-regulation, the level of which was reduced in TPH1$^{-/-}$ mice with delayed Hes1 up-regulation (Fig. 6C). This suggests that the inhibitory function of Hes1 on Ptf1a expression described during development may be active also in the adult pancreas. We further explored the Hes1-Ptf1a regulation in an *in vitro* system where the acinar cell line AR42J was stimulated toward a more differentiated and secretory phenotype by treatment with dexamethasone [37]. 48 hours of dexamethasone treatment changed the cell morphology from a more spheroid to flattened shape (Fig. S9A), led to decreased replication of AR42J cells (Fig. S9B), increased amylase content (Fig. 6D) and decreased Sox-9 expression (Fig. 6E). Similar to what we observed *in vivo*, AR42J differentiation showed an initial increase of Ptf1a expression that was then reduced following Hes1 up-regulation (Fig. 6F).

Taken together, the *in vivo* and *in vitro* results presented here show that conditions of increased zymogen secretion and synthesis in acinar cells are associated with the activation of the Hes1-Ptf1a axis. These data not only indicate that different progenitor genes are regulated in a different manner in acinar cells, but also that the Hes1-Ptf1a axis may contribute to maintain physiological zymogen levels in the adult pancreas in the absence of acinar cell damage.

**Discussion**

The remarkable regenerative capacity observed in organs of several non-mammalian vertebrates often involves the de-differentiation of mature cells (reviewed in [38]). Notably, cell de-differentiation is also observed in the regeneration of the adult exocrine pancreas of mammals following pancreatitis. In this organ, differentiated acinar cells transiently revert into a progenitor state following injury, thus likely acting as facultative progenitor cells and initiating a replicative program leading to exocrine tissue repair (reviewed in [1,39,40]). This de-differentiation of adult pancreatic acinar cells is a critical process within the regenerative mechanism, as *bona fide* resident stem cells have not been reported in the adult pancreas (reviewed in [39]). Our results showed that, differently from what has been observed in other cell types and tissues [6,33,41-46], 5-HT does not act as a strong mitogen for acinar cells, as acinar replication was unchanged *in vivo* in the absence of 5-HT following 60% pancreatectomy and T3 stimulation and *in vitro* in AR42J acinar cells treated with 5-HT (data not shown). However, 5-HT is required for acinar de-differentiation following inflammatory-mediated tissue damage. In this context, it is important to mention that 60% pancreatectomy is characterized by a moderate level of acinar cell de-differentiation and replication compared with the more robust regeneration observed during induction of pancreatitis. These observations support the notion that, according to the type and extent of tissue injury, the mammalian pancreas has the ability to trigger different regenerative mechanisms, which are driven by activation of a progenitor-like program or by a simple, clonal proliferation of differentiated acinar cells (reviewed in [39,47]). Furthermore, as progenitor-based and clonal modalities of regeneration are not mutually exclusive and are likely to co-exist, the clonal regeneration independent from zymogen secretion is likely to account for the replication observed in TPH1$^{-/-}$ mice with compromised progenitor-based regeneration.

A major challenge undertaken in our study was to elucidate the mechanisms by which 5-HT affects acinar de-differentiation and ADM formation. We identified 5-HT-dependent secretion of zymogen content as one of the step promoting up-regulation of progenitor-like genes, suggesting that physical removal of zymogens via secretion has to occur to allow de-differentiation of adult acinar cells. However, additional mechanisms are

likely to contribute to the observed delayed de-differentiation and ADM formation in the absence of 5-HT. We found that reduced secretion in TPH1$^{-/-}$ mice limits chemokine production and leukocyte infiltration in the early stages of cerulein-induced pancreatitis ([10] and this work). Thus, it is possible that the few infiltrating leukocytes released a limited amount of pro-inflammatory cytokines that may not have been sufficient to stimulate acinar de-differentiation and ADM formation. Indeed, TPH1$^{-/-}$ mice had reduced levels of TNF$\alpha$ and MMP, both factors critical to induce ADM formation [28]. The comparable de-differentiation observed *in vivo* in isolated acini supports the hypothesis that the 5-HT effect is not acinar cell autonomous but is mediated by macrophage-stimulated extracellular matrix digestion. However, it has to be noted that, despite sharing phenotypic similarities, the *in vitro* acinar de-differentiation may not completely reflect the molecular mechanisms occurring in the *in vivo* de-differentiation upon pancreatitis induction. Indeed, the process of acinar isolation *per se* activates Ras and its down-stream effectors ERK and AKT [29], the activity of which promotes cell de-differentiation. Thus, it is possible that the *in vitro* procedure bypasses a cell autonomous, 5-HT-mediated process occurring *in vivo*. In support of this hypothesis, 5-HT has been reported to act upstream Ras-dependent ERK1/2 activation via binding to different 5-HT receptors [48-50].

In this regard, an alternative explanation for the aberrant progenitor-based regeneration observed in TPH1$^{-/-}$ mice is that 5-HT directly stimulates the expression of progenitor genes via 5-HT receptor signaling or intracellular mechanisms following its uptake. Of note, type-1 5-HT receptors were recently described to promote proliferation of human pancreatic cancer cells [34]. Type 1 and 2 5-HT receptors were up-regulated during pancreatic regeneration and we also observed a robust up-regulation of type-2 5-HT receptors concomitant with the progenitor gene Hes1 expression following PPX. Finally, we also discovered an activation of the Hes1-Ptf1a axis in the uninjured pancreas. Both under *in vivo* and *in vitro* experimental conditions, Hes1 up-regulation was accompanied by down-regulation of Ptf1a expression. This suggests the presence of a tightly regulated control of Ptf1 expression by Hes1 in the adult pancreas, reminiscent of the embryonic situation. Hence, our data are in support of a dual role of Hes1 in the pathophysiology of the pancreas. In physiological situations, increased zymogen secretion is associated with moderate Hes1 up-regulation, which limits Ptf1a expression and thus contributes to acinar homeostasis by preventing excessive zymogen expression. In pathological situations that trigger acinar replication, a higher Hes1 up-regulation occurs with a consequent reduction of transcription factors and proteins required to maintain acinar function. This down-regulation of differentiation genes may provide the cells with the suitable environment to up-regulate a broader set of progenitor genes, leading to a robust acinar proliferation and organ repair. Collectively, these results indicate that expression of progenitor genes is not a synchronous event. Therefore, similarly to what was observed in the determination of diverse cell fates during development [56], the different time of expression of these transcription factors in the adult pancreas may reflect their distinctive function during homeostasis and regeneration.

While highlighting the impact that 5-HT exerts on the de-differentiation and proliferation abilities of pancreatic cells under different regenerative conditions, this study includes several limitations. First, the use of a general TPH1 knocked out model system precludes the dissection of the individual cell contribution in the synthesis of 5-HT. In addition, further studies are necessary to characterize the role of the different 5-HT receptors present on acinar and non-acinar cells to fully understand their contribution during pancreatic regeneration. Finally, an aspect of the 5-HT biology that has not been addressed in this study is a thorough characterization of vascular function. Disturbances in microcirculation, including vasoconstriction, are associated with acute pancreatitis (reviewed in [57]). As 5-HT is a potent vasoconstrictor, it will be worth exploring whether alterations in the vascular biology contribute to the observed phenotype in TPH1$^{-/-}$ mice.

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**Contributors** The authors of this manuscript contributed in the study design, acquisition, analysis, interpretation of data, drafting and critical revision of the manuscript. ES, KG, MB, RB, ABS, EM, GS performed experiments, generated and analyzed data; ABH performed confocal microscopy, generated and analyzed data; EMS performed electron microscopy; YT performed partial pancreatectomy; AZ performed experiments and generated data; TR generated transgenic lines; RG, SS designed the study; ES, SS wrote the manuscript; RG, ABS revised the manuscript for content, analysis and interpretation of data. All authors approved the submitted version.

**List of abbreviations** ADM, acinar-to-ductal metaplasia; TPH1$^{-/-}$, tryptophan hydroxylase 1 knocked-out; PPX, 60% partial pancreatectomy; 5-HTP, 5-hydroxytryptophan; T3, 3,5,3-L-tri-iodothyronine.

**List of online supporting material:**

Supplementary material and methods and four supplementary figures

**Figure legends**

**Figure 1**. Lack of 5-HT results in defective acinar cell proliferation and delayed ADM formation. **A**. Quantification of replication markers expressed in pancreatic acinar cells showed increased number of Ki67 positive cells in TPH1$^{-/-}$ mice after 7 and 14 days of pancreatitis. **B**. Relative amount of acinar cells in G2 and M phases determined by pH3 staining pattern 7 and 14 days after pancreatitis induction. Data are expressed as percentage of cells with punctated (G2 phase) or complete nuclear staining (M phase) out of the total number of pH3 positive cells. **C**. Hematoxylin-Eosin (H&E)-staining and ADM quantification showed lower amount of ADM (asterisks) in TPH1$^{-/-}$ mice after three days of pancreatitis induction. Lower panels, enlarged views of insets three days after pancreatitis. Note the presence of mature ADM in WT mice characterized by loss of acinar content and tubular complex formation. TPH1$^{-/-}$ pancreata presented sporadic de-regulation of acinar content (stars) without re-organization into tubular structures. **D**. Confocal pictures showing nuclear localization of β-catenin in WT and TPH1$^{-/-}$ pancreata three days after induction of pancreatitis. Nuclei are stained with DAPI (blue). **E**. Amylase and β-catenin staining three days after induction of pancreatitis. Circled area and asterisk in WT pancreata represent ADM. Right panels, enlarged views of insets. Arrowhead, area with reduced amylase content and increased β-catenin expression in TPH1$^{-/-}$ pancreata. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

**Figure 2**. Lack of 5-HT delays acinar de-differentiation during pancreatitis. **A**. Cytokeratin 19 (CK19), Sox9, β-catenin and amylase staining three and seven days after induction of pancreatitis. Nuclei in the IF pictures are stained with DAPI (blue). **B**. qPCR of ductal and progenitor markers during the indicated times of cerulein-induced pancreatitis showed delayed expression in TPH1$^{-/-}$ pancreata. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

**Figure 3**. Lack of 5-HT prevents loss of amylase during pancreatitis. Biochemical determination of amylase activity (**A**) and immunoblotting of 10 μg of proteins (**B**) showed down-regulation of amylase in pancreatic tissue of WT but not TPH1$^{-/-}$ mice one day after cerulein treatment. **C**. Immunostaining of amylase during 14 days of pancreatitis induction showed decreased protein expression in WT but not in TPH1$^{-/-}$ mice one day after cerulein treatment. **D**. Electron micrographs of WT and TPH1$^{-/-}$ pancreata. Note the close proximity of zymogen granules with reduced inter-granule space in TPH1$^{-/-}$ mice, both in control and cerulein treatment. L, acinar lumen with evident microvilli. V, zymogen vesicle, M, mitochondrion. RER, rough endoplasmic reticulum. Right panel, quantification of the area occupied by granules expressed as percentage of total area. **E**. Size distribution of zymogen vesicle diameter. Note the higher abundance of vesicles with large diameter in TPH1$^{-/-}$ mice following pancreatitis induction. Results are average ± SEM (n=5), *p<0.05. Scale bars: 1 μm.

**Figure 4**. **A**. Scheme depicting the combined regimen with 5-HTP and cerulein (Cer). 0.9% NaCl was used as vehicle control. Circles indicate days of treatment starting from the first cerulein injection and triangles indicate days of animal harvest. Pan-leukocyte immunostaining with anti-coronin 1 (**B**) and qPCR of pro-inflammatory chemokine MCP-1 (**C**) showed lower inflammation upon 5-HTP supplementation one day after induction of pancreatitis. Serum (**D**) and tissue (**E**) levels of amylase 8 hours after pancreatitis induction following 5-HTP supplementation. qPCR of Hes1 (**F**) and immunostaining of Sox9 (**G**) one day after pancreatitis induction following 5-HTP supplementation. **H**. inflammarory cell quantification after PU.1 immunostaining showed reduced infiltration in TPH1$^{-/-}$ mice three days after pancreatitis induction. **I**. qPCR of type1 and 2 macrophage markers three days after pancreatitis induction. **L**. qPCR of matrix metalloproteinase 7 (MMP7) at the indicated days of pancreatitis induction. **M**. qPCR of progenitor and zymogen markers in isolated acini (inset) cultured for 24 hours in suspension to induce differentiation. 5-HT was added to the medium at 20 μM concentration. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

**Figure 5**. Lack of 5-HT does not alter clonal regeneration of acinar cells following 60% pancreatectomy (PPX) and thyroid hormone T3 stimulation. **A**. Quantification of replication markers expressed in pancreatic acinar cells at the indicated times after partial pancreatectomy. **B**. Immunostaining of Ki67 at the indicated time points following PPX. Pictures represent an inset (1:10) of the original images used for Ki67 quantification. **C**. qPCR of progenitor-cell markers at the indicated times after PPX. Note that WT but not TPH1$^{-/-}$ mice up-regulated Hes1

expression 14 days after surgery. Expression levels obtained in WT mice after cerulein induced pancreatitis (WT CIP) are shown as a comparison. **D**. Immunostaining of amylase showed comparable protein expression in WT and TPH1$^{-/-}$ mice following PPX. **E**. Quantification of Ki67 expressed in pancreatic acinar cells following T3 administration for six days. **F**. Pan-leukocyte immunostaining with anti-coronin1 showed comparable level of inflammatory cells in control and treated mice. Results are average ± SEM (n=3-5), *p<0.05. Scale bars: 50 μm.

**Figure 6**. Increased zymogen secretion stimulates Hes1 expression in the uninjured pancreas. **A**. Left panel, immunostaining of Sox9 following 5-HTP supplementation showed nuclear expression of the protein in interstitial and centro-acinar cells but not in acinar cells. Right panel, qPCR analysis showed comparable levels of Sox9 transcripts in control and 5-HTP-treated pancreata. **B**. qPCR analysis showed Hes-1 up-regulation and Ptf1a down-regulation in mouse pancreata following 5-HTP treatment. **C**. qPCR of Ptf1a and Hes1 expression in WT and TPH1$^{-/-}$ mice in the early phases after pancreatitis induction. Dexamethasone treatment of pancreatic acinar AR42J cells for 48 hours increased cellular content of amylase (**D**) and reduced Sox9 expression at both transcript (left panel) and protein (right panel) levels (**E**), indicative of increased cellular differentiation. **F**. qPCR analysis showed a time-dependent increase of Hes1 and decrease of Ptf1a expression in dexamethasone-treated cells. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.


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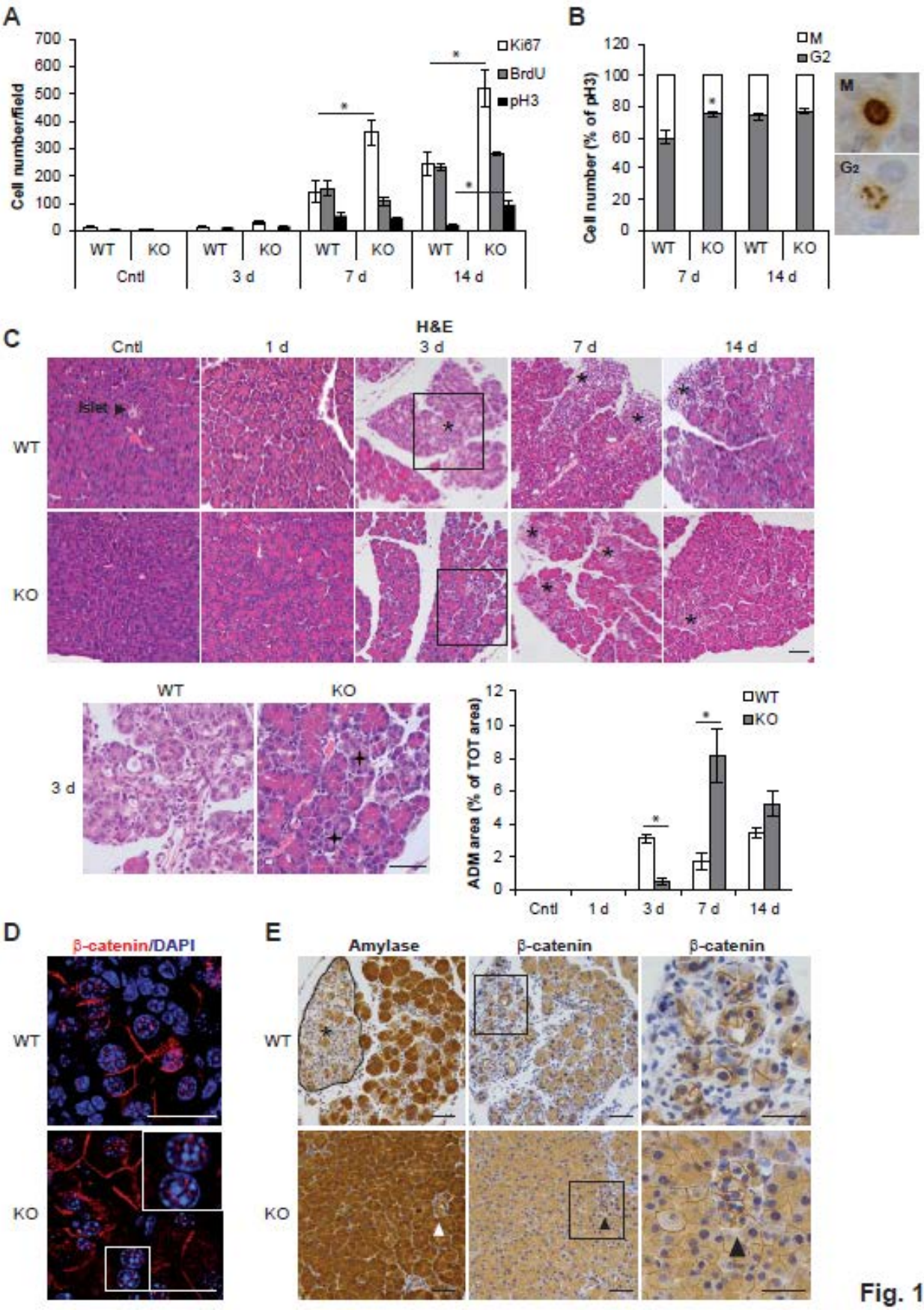
A
Cell number/field
Ki67
BrdU
pH3
WT
KO
Cntl
3 d
7 d
14 d
B
Cell number (% of pH3)
M
G2
M
G2
C
H&E
Cntl
1 d
3 d
7 d
14 d
Islet
WT
KO
ADM area (% of TOT area)
D
β-catenin/DAPI
E
Amylase
β-catenin

Fig. 1

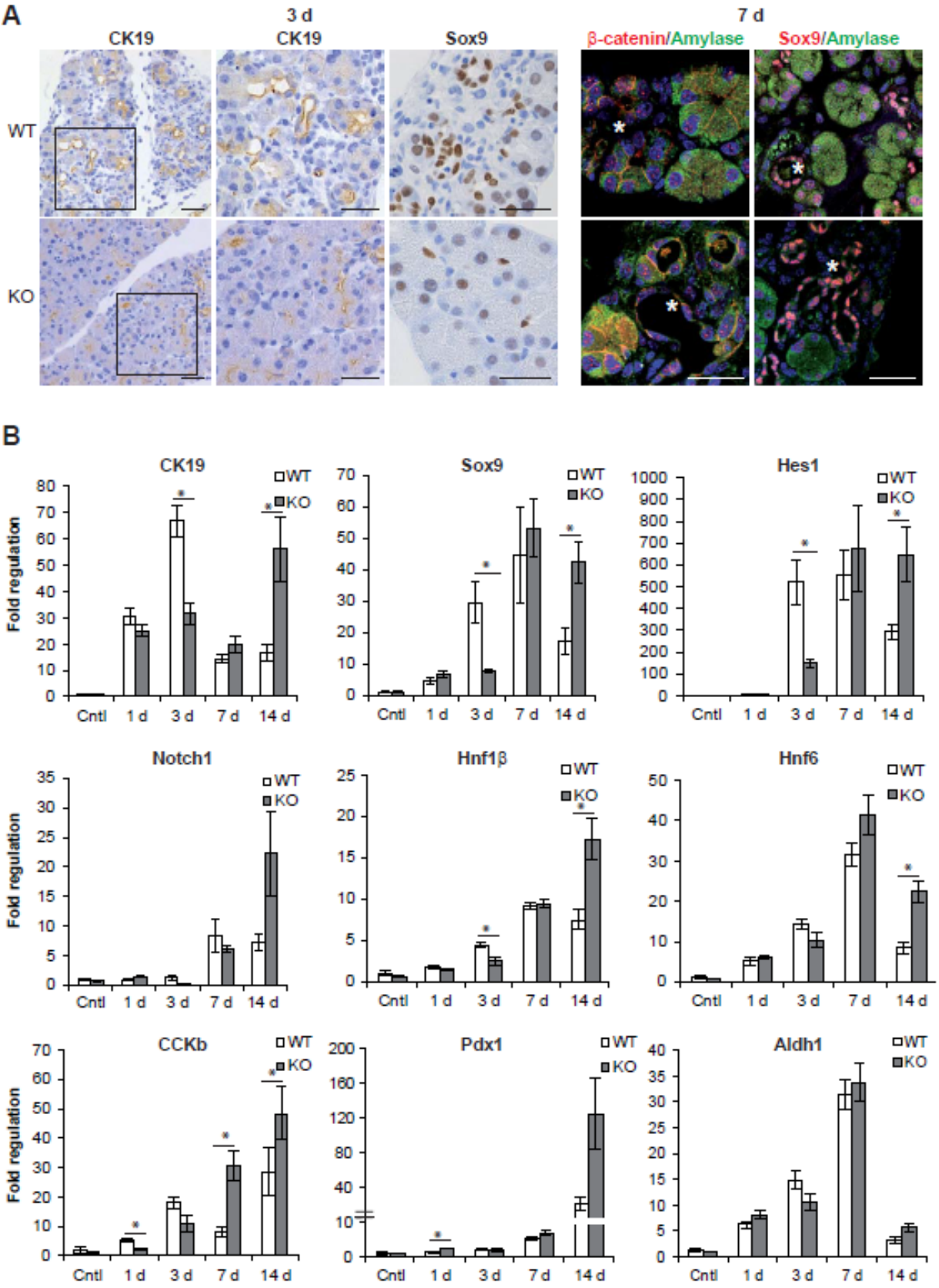
A
3 d
7 d
CK19
CK19
Sox9
β-catenin/Amylase
Sox9/Amylase
WT
KO
B
CK19
Sox9
Hes1
Notch1
Hnf1β
Hnf6
CCKb
Pdx1
Aldh1
Fold regulation
WT
KO
Cntl
1 d
3 d
7 d
14 d

Fig. 2

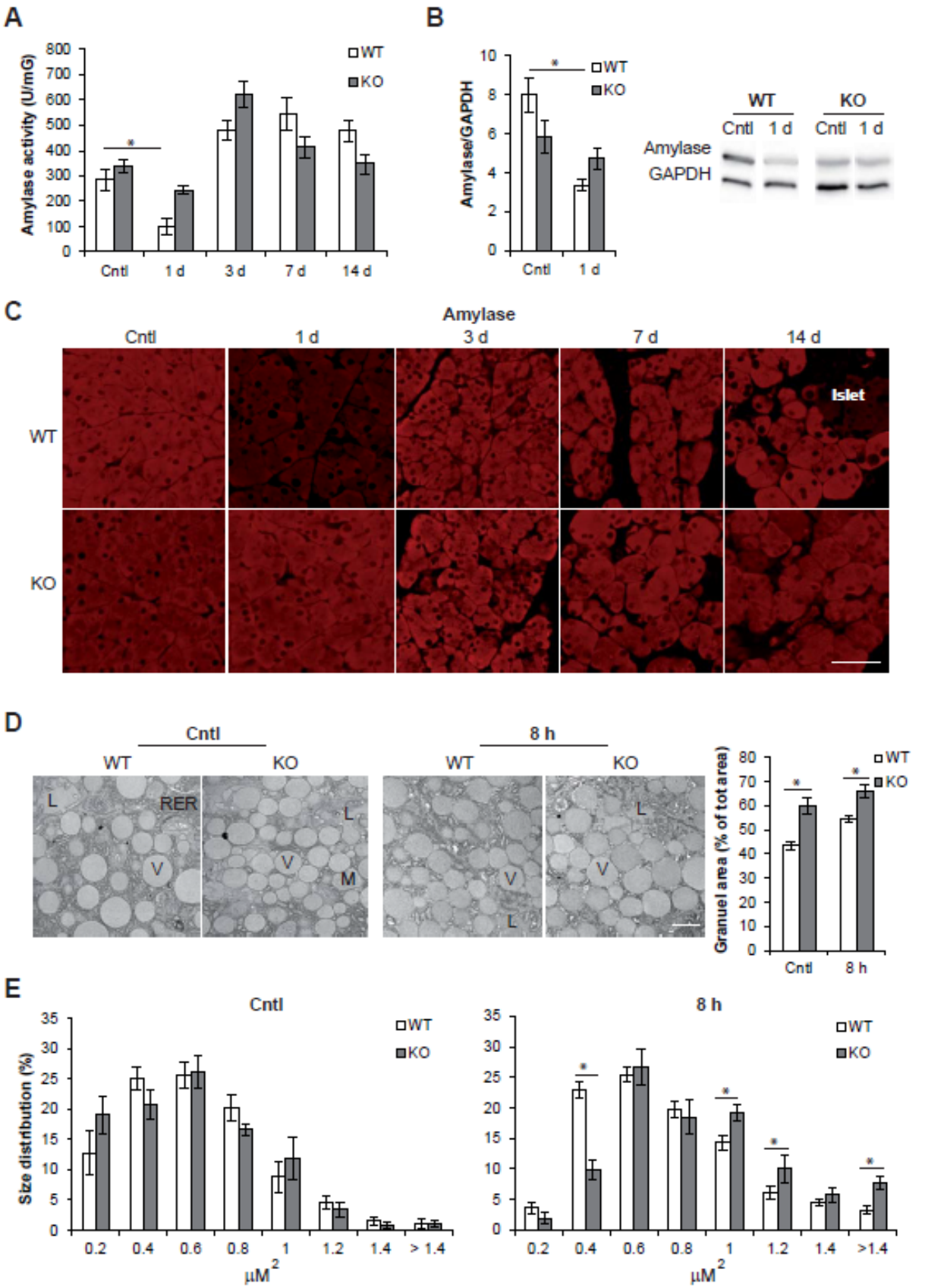
A
Amylase activity (U/mG)
800
700
600
500
400
300
200
100
0
WT
KO
*
Cntl
1 d
3 d
7 d
14 d
B
Amylase/GAPDH
10
8
6
4
2
0
*
WT
KO
Cntl
1 d
WT
KO
Cntl
1 d
Cntl
1 d
Amylase
GAPDH
C
Amylase
Cntl
1 d
3 d
7 d
14 d
WT
KO
Islet
D
Cntl
8 h
WT
KO
WT
KO
L
RER
V
L
V
M
WT
KO
L
V
L
V
L
Granuel area (% of tot area)
80
70
60
50
40
30
20
10
0
*
*
WT
KO
Cntl
8 h
E
Cntl
8 h
Size distribution (%)
35
30
25
20
15
10
5
0
WT
KO
*
*
*
*
0.2
0.4
0.6
0.8
1
1.2
1.4
> 1.4
>1.4
µM2

Fig. 3

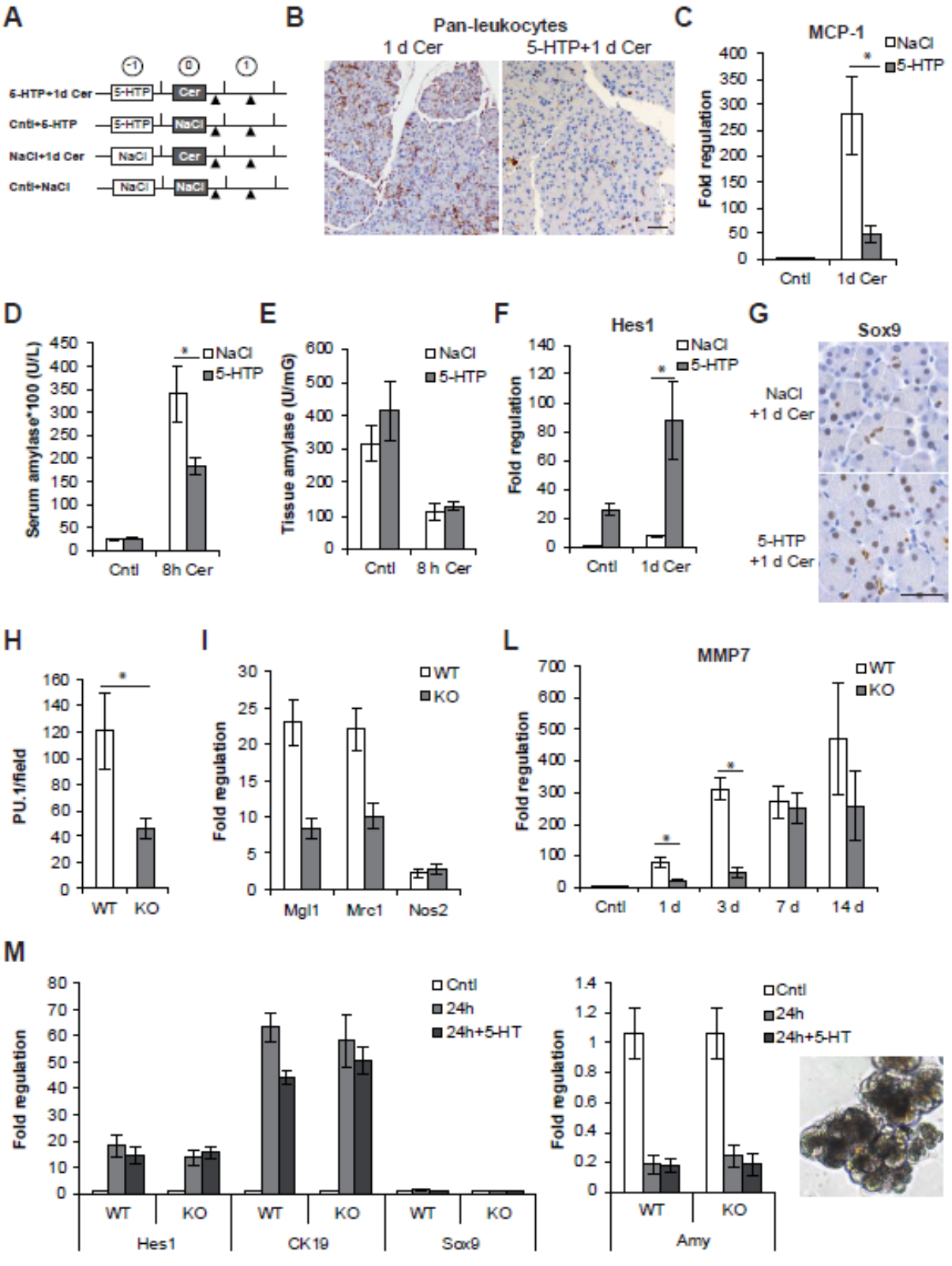
A
-1
0
1
5-HTP+1d Cer
5-HTP
Cer
Cntl+5-HTP
5-HTP
NaCl
NaCl+1d Cer
NaCl
Cer
Cntl+NaCl
NaCl
NaCl
B
Pan-leukocytes
1 d Cer
5-HTP+1 d Cer
C
MCP-1
NaCl
5-HTP
Fold regulation
Cntl
1d Cer
D
Serum amylase*100 (U/L)
NaCl
5-HTP
Cntl
8h Cer
E
Tissue amylase (U/mG)
NaCl
5-HTP
Cntl
8 h Cer
F
Hes1
NaCl
5-HTP
Fold regulation
Cntl
1d Cer
G
Sox9
NaCl
+1 d Cer
5-HTP
+1 d Cer
H
PU.1/field
WT
KO
I
WT
KO
Fold regulation
Mgl1
Mrc1
Nos2
L
MMP7
WT
KO
Fold regulation
Cntl
1 d
3 d
7 d
14 d
M
Cntl
24h
24h+5-HT
Fold regulation
WT
KO
Hes1
CK19
Sox9
Cntl
24h
24h+5-HT
Fold regulation
WT
KO
Amy

Fig. 4

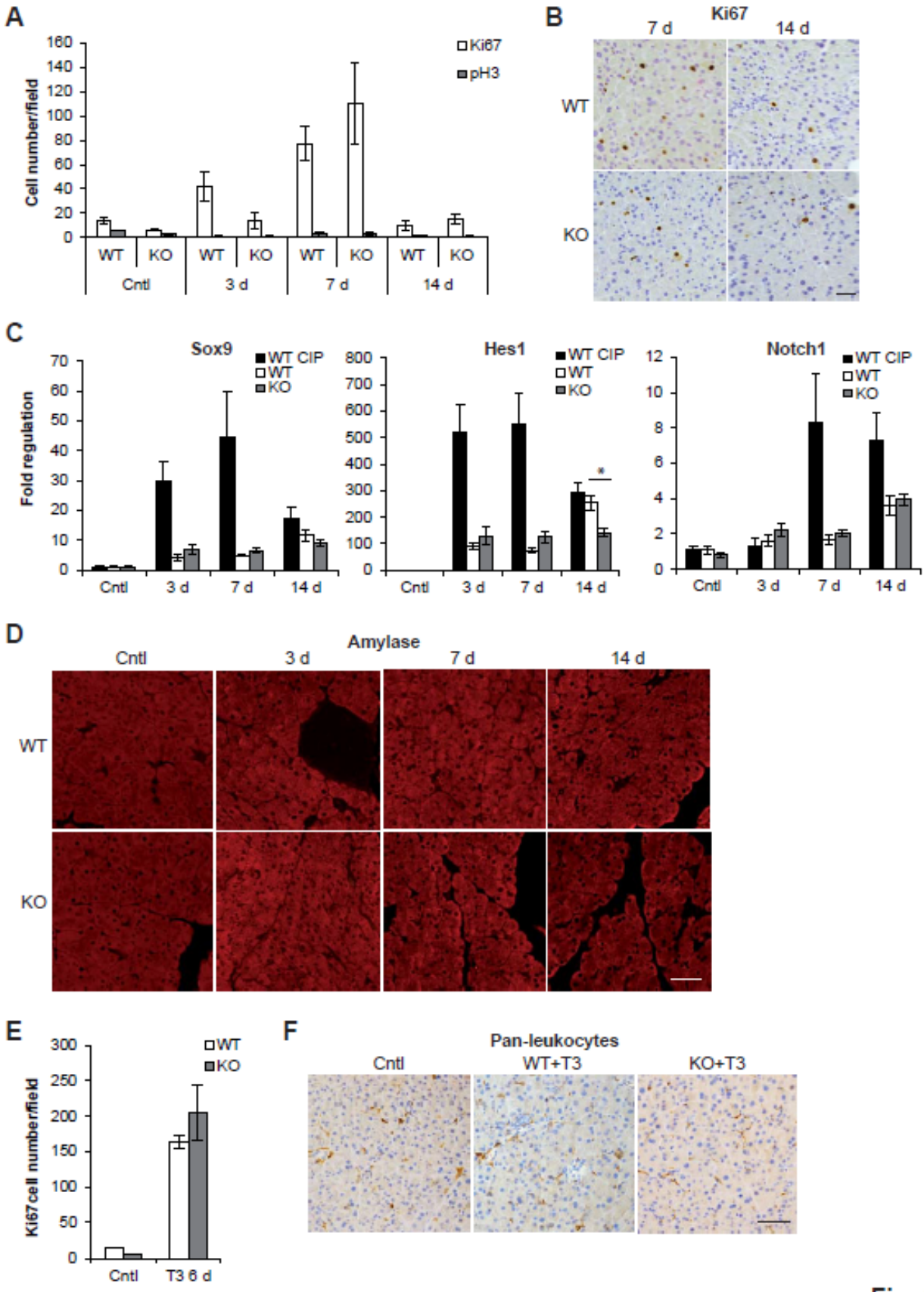
A
Cell number/field
Ki67
pH3
WT
KO
Cntl
3 d
7 d
14 d
B
Ki67
7 d
14 d
WT
KO
C
Sox9
Hes1
Notch1
WT CIP
WT
KO
Fold regulation
Cntl
3 d
7 d
14 d
D
Amylase
Cntl
3 d
7 d
14 d
WT
KO
E
Ki67cell number/field
WT
KO
Cntl
T3 6 d
F
Pan-leukocytes
Cntl
WT+T3
KO+T3

Fig. 5

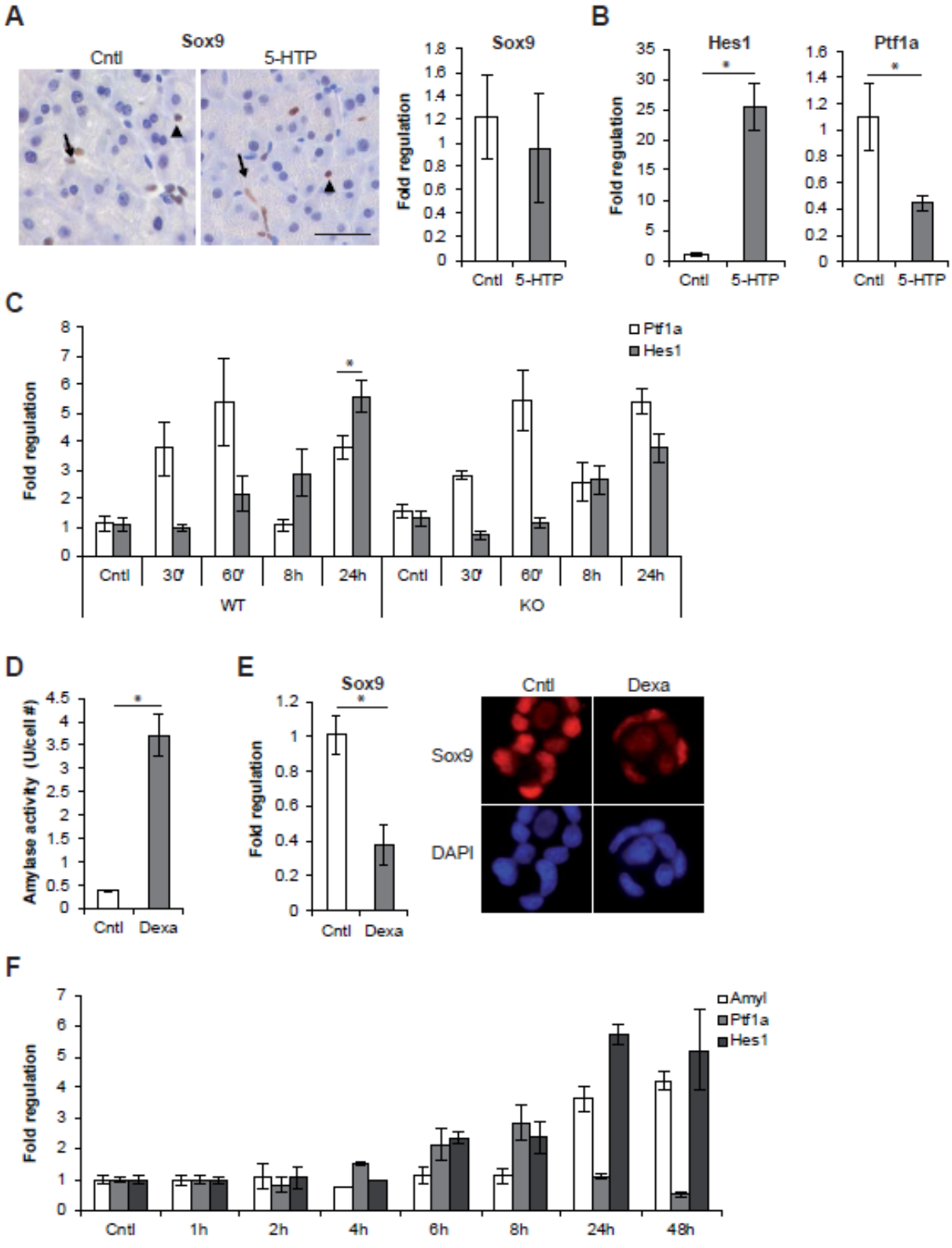
A
Sox9
Cntl
5-HTP
Sox9
Fold regulation
Cntl
5-HTP
B
Hes1
Ptf1a
Fold regulation
Cntl
5-HTP
C
Ptf1a
Hes1
Fold regulation
Cntl
30'
60'
8h
24h
WT
KO
D
Amylase activity (U/cell #)
Cntl
Dexa
E
Sox9
Fold regulation
Cntl
Dexa
Cntl
Dexa
Sox9
DAPI
F
Amyl
Ptf1a
Hes1
Fold regulation
Cntl
1h
2h
4h
6h
8h
24h
48h

**Fig. 6**

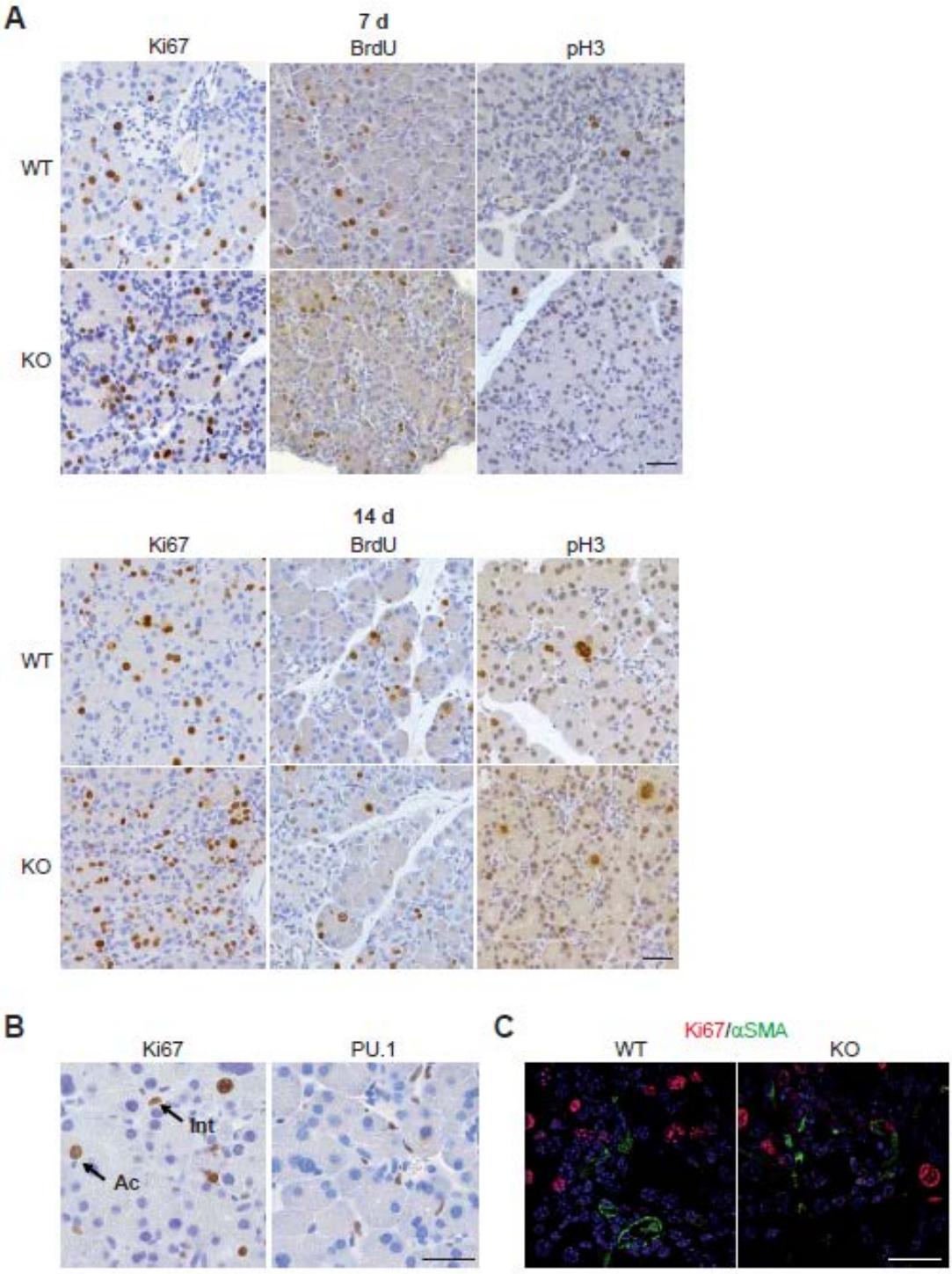
A
7 d
Ki67
BrdU
pH3
WT
KO
14 d
Ki67
BrdU
pH3
WT
KO
B
Ki67
PU.1
Int
Ac
C
Ki67/αSMA
WT
KO

**Fig. S1**

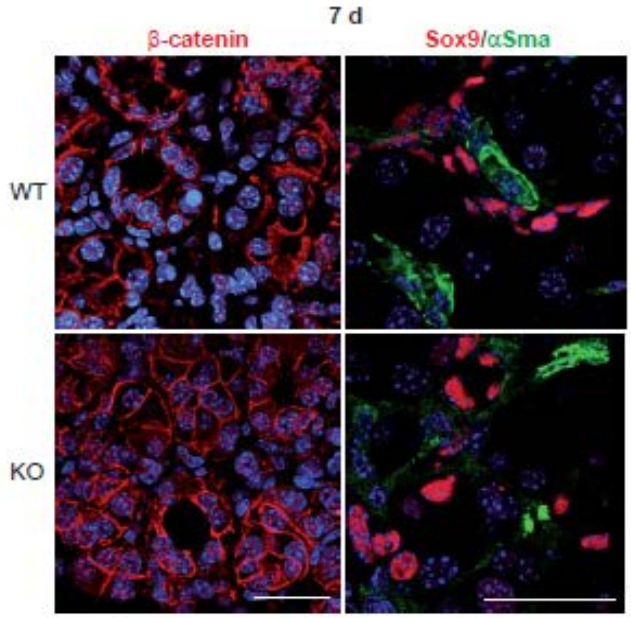


Fig. S2

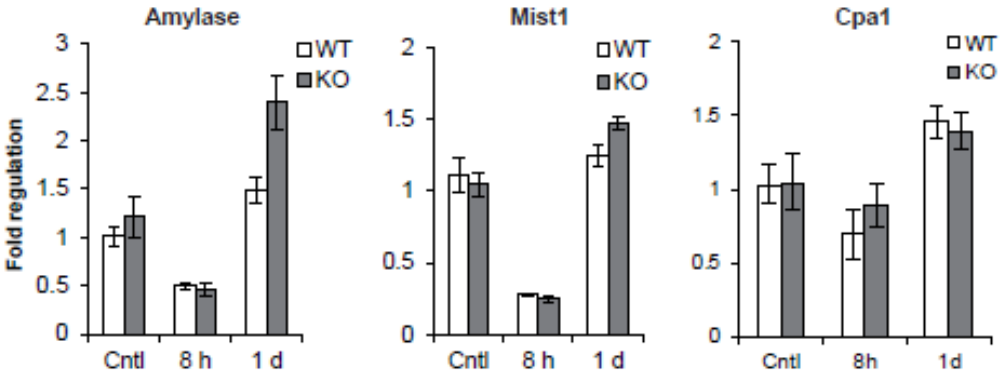
Amylase
Mist1
Cpa1
WT
KO
Fold regulation
3
2.5
2
1.5
1
0.5
0
Cntl
8 h
1 d
8h
1d

**Fig. S3**

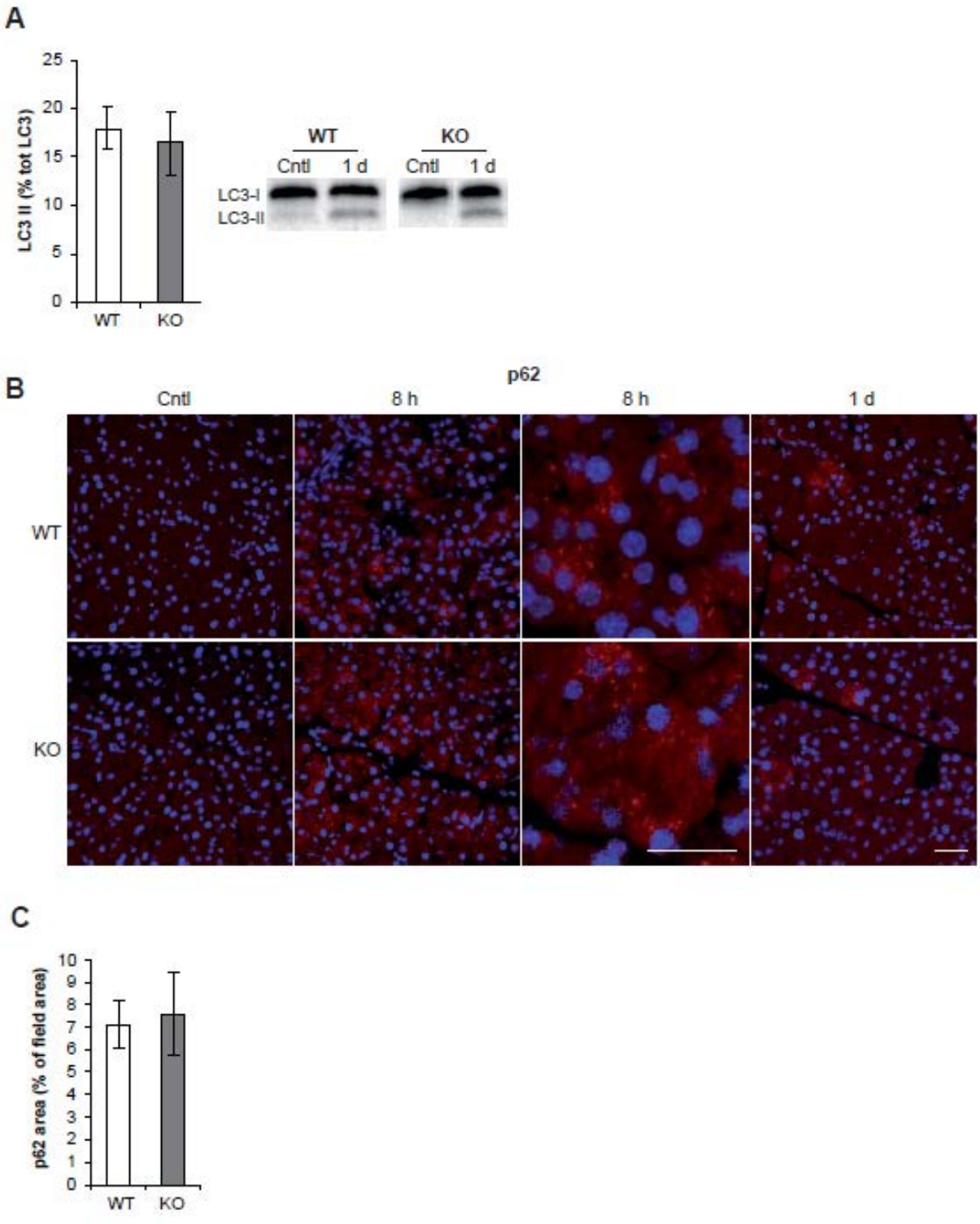
A
LC3 II (% tot LC3)
25
20
15
10
5
0
WT
KO
WT
KO
Cntl
1 d
Cntl
1 d
LC3-I
LC3-II
B
p62
Cntl
8 h
8 h
1 d
WT
KO
C
p62 area (% of field area)
10
9
8
7
6
5
4
3
2
1
0
WT
KO

**Fig. S4**

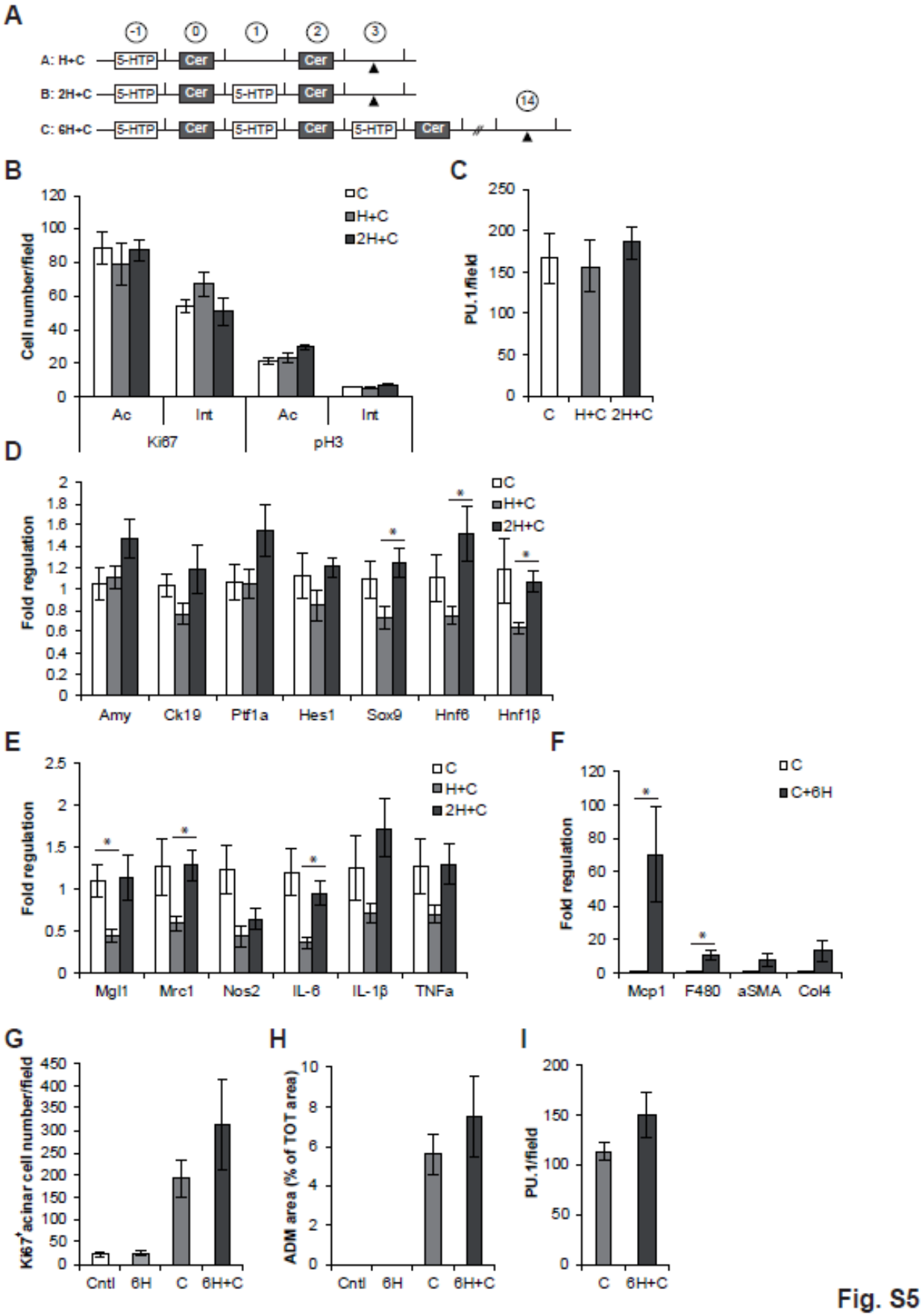
A
-1
0
1
2
3
14
A: H+C
B: 2H+C
C: 6H+C
5-HTP
Cer
B
Cell number/field
C
H+C
2H+C
Ac
Int
Ki67
pH3
C
PU.1/field
D
Fold regulation
Amy
Ck19
Ptf1a
Hes1
Sox9
Hnf6
Hnf1β
E
Mgl1
Mrc1
Nos2
IL-6
IL-1β
TNFa
F
C+6H
Mcp1
F480
aSMA
Col4
G
Ki67+ acinar cell number/field
Cntl
6H
C
6H+C
H
ADM area (% of TOT area)
I
PU.1/field

Fig. S5

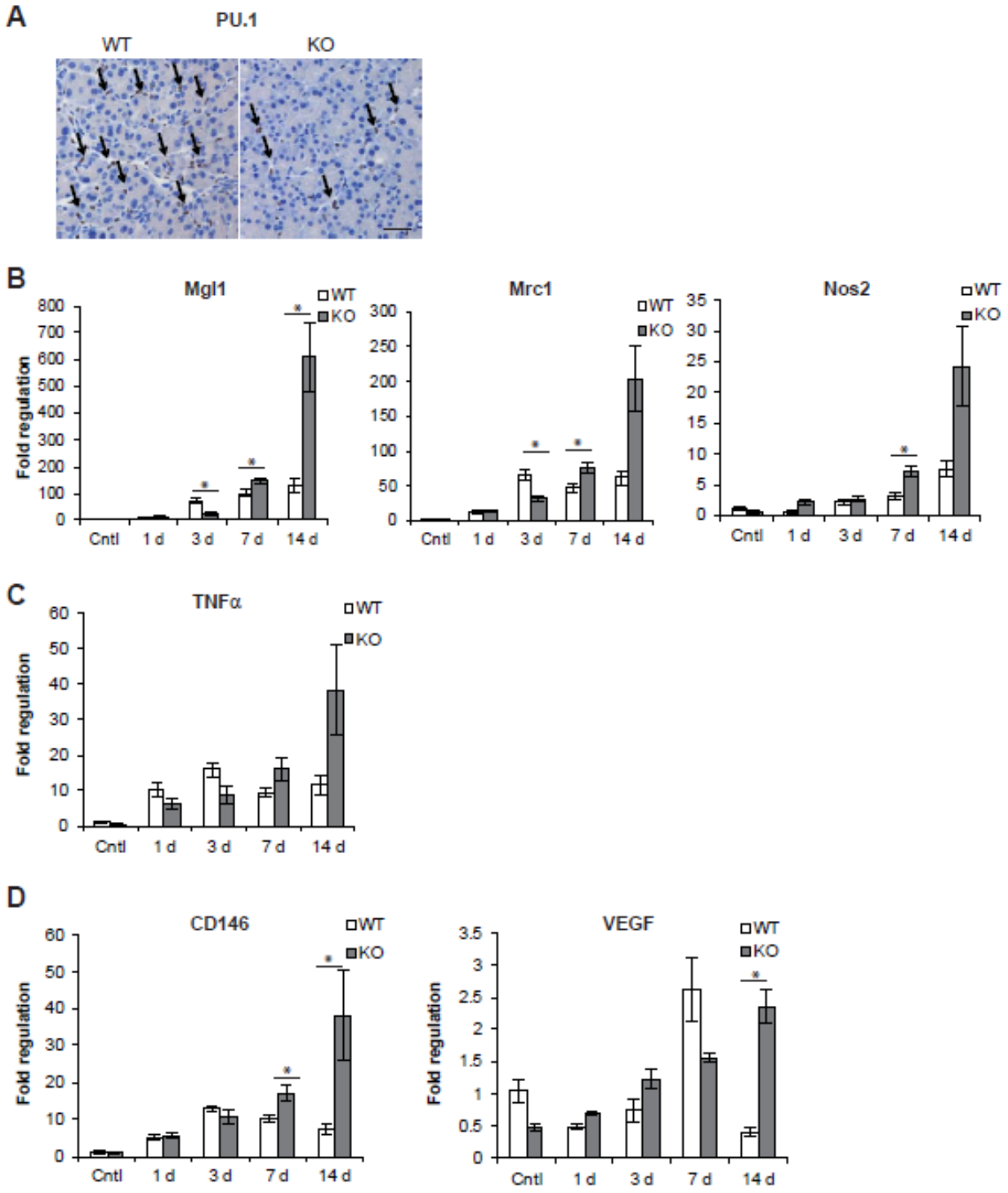
A
PU.1
WT
KO
B
Mgl1
Mrc1
Nos2
WT
KO
Fold regulation
Cntl
1 d
3 d
7 d
14 d
C
TNFα
D
CD146
VEGF

Fig. S6

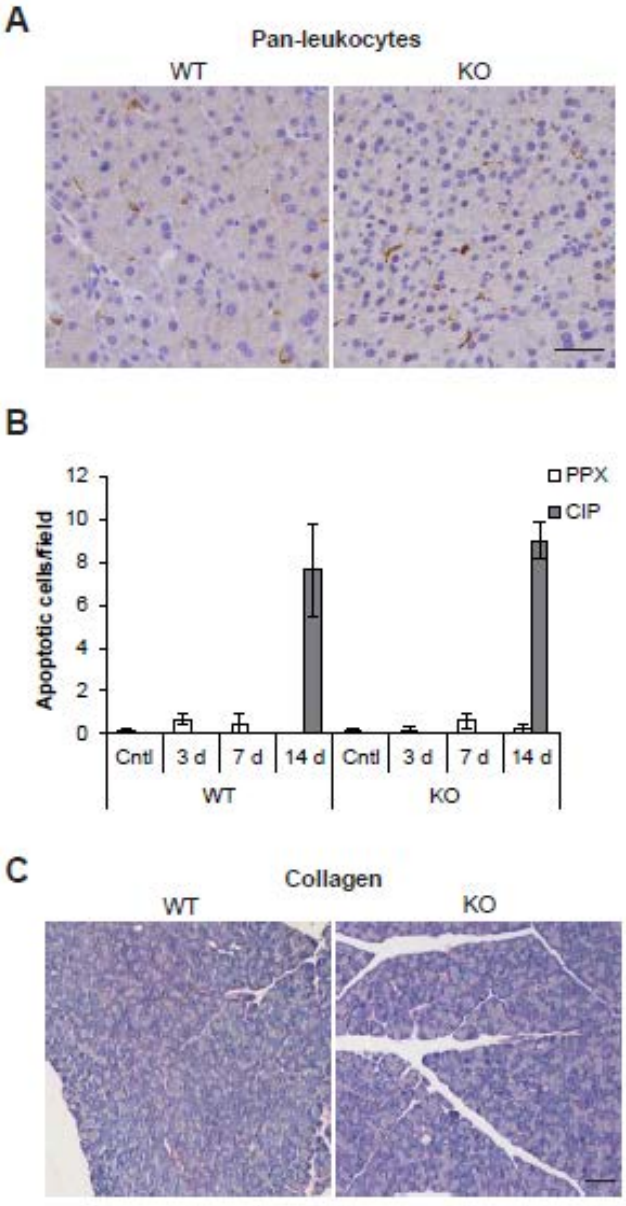
A
Pan-leukocytes
WT
KO
B
PPX
CIP
Apoptotic cells/field
12
10
8
6
4
2
0
Cntl
3 d
7 d
14 d
Cntl
3 d
7 d
14 d
WT
KO
C
Collagen
WT
KO

**Fig. S7**

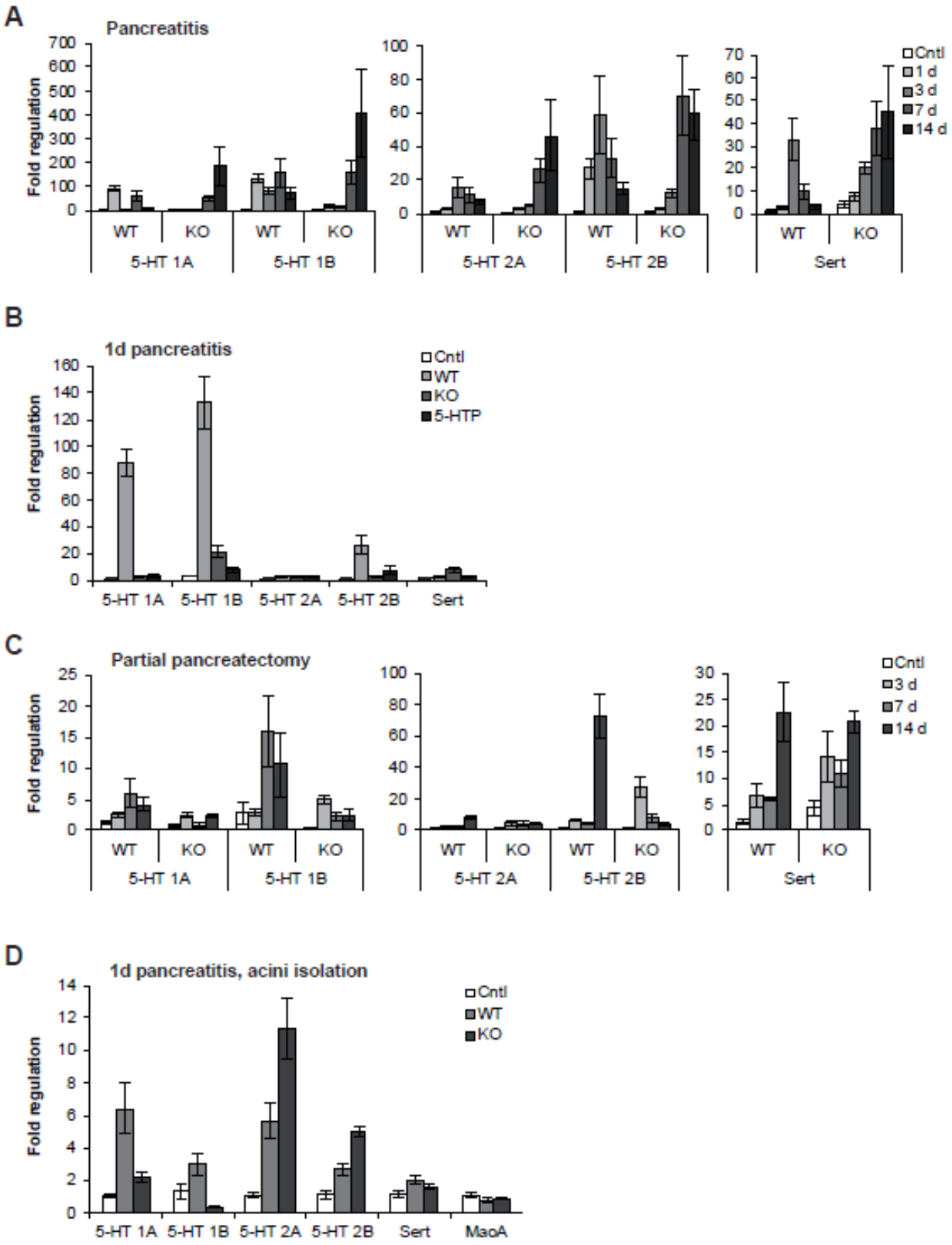
A
Pancreatitis
Fold regulation
WT
KO
5-HT 1A
5-HT 1B
5-HT 2A
5-HT 2B
Sert
Cntl
1 d
3 d
7 d
14 d
B
1d pancreatitis
Cntl
WT
KO
5-HTP
C
Partial pancreatectomy
D
1d pancreatitis, acini isolation
MaoA

Fig. S8

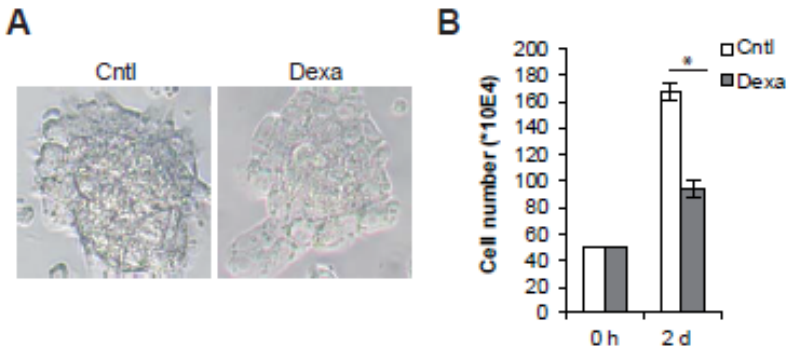
A
Cntl
Dexa
B
Cell number (*10E4)
200
180
160
140
120
100
80
60
40
20
0
0 h
2 d
*
Cntl
Dexa

**Fig. S9**

**Supplementary Online Materials**

**Materials and Methods**

***Biochemical reagents***

Unless otherwise stated, all chemicals were purchased from Sigma and cell culture reagents from Gibco-BRL. Stock solution for 5-HTP and cerulein were made in NaCl, dexamethasone in F-12K medium.

***Pancreatitis induction***

Animals received six hourly injections of 50 μg/kg cerulein on Monday, Wednesday and Friday and were harvested on Tuesday after one set of injections (day 1), on Thursday after two sets of injections (day 3) or on Monday after three (day 7) or six (day 14) sets of injections, without receiving cerulein treatment on the same day.

***Pancreatic cell culture***

Pancreatic acini were isolated from WT and TPH1$^{-/-}$ mice was performed as previously described in [1]. Cells de-differentiaion was induced according to [2] by culturing isolated acini in suspension for 24 hours in RPMI 1640 glutamax medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 0.1 mg/ml soybean trypsin inhibitor. ******

AR42J cells were maintained in Kaighn's modified Ham's F-12 medium with 20% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a 5% $CO_2$ atmosphere. Differentiation was induced by incubating adherent cells with 50 nM dexamethasone.

***Biochemical analysis of amylase activity***

For determination of amylase levels present in the serum, blood was sampled by heart puncture. For determination of amylase levels present in pancreatic tissue, pancreata were homogenized with RIPA buffer containing protease inhibitor cocktail (complete ultra-tablets mini, Roche Diagnostics, Mannheim, Germany). Amylase activity was measured using the Fuji Dri-Chem 4000i analyzer (Glattbrugg, Switzerland).

***Immunohistochemistry***

Pancreas specimens were embedded in paraffin for histological analyses as described [3]. For immunofluorescence analysis of cultured cell lines, AR42J cells were fixed in 3.6% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS. Primary antibodies used in this study were: rabbit anti-phospho-histone 3 (Millipore, Massachusetts, USA), rabbit anti-sox9 (Millipore, Massachusetts, USA), rabbit anti-cyclin E (Abcam, Cambridge, UK), rabbit anti-coronin 1 (gift from Jean Pieters), rabbit anti-amylase (Sigma-Aldrich, Buchs, Switzerland), rabbit anti Ki67 (Abcam, Cambridge, UK), rabbit anti-p62/SQSTM1 (MBL, Massachusetts, USA), rabbit anti-β-catenin (Cell Signaling, Danvers, USA) , rabbit anti-Pu1 (Cell Signaling, Danvers, USA), mouse anti-αSMA (Dako, Glostrup, Denmark), goat anti-amylase (Santa Cruz Biotechnology, California).. Secondary antibodies used in this study were: Biotinylated Goat Anti-Rabbit IgG (H+L). This antibody is included in the VECTASTAIN® ABC kits, AlexaFluor 594, Goat Anti-Rabbit IgG (Life Technologies, Carlsbad, California, USA), FITC anti goat (***), FITC anti mouse (***). Nuclei were visualized with 4′, 6-diamidino-2-phenylindole (DAPI). Detection of proliferating cells in s-phase was performed with Invitrogen BrdU Staining Kit (Life Technologies, Carlsbad, California, USA). Detection of DNA fragmentation in apoptotic cells was performed with a TUNEL assay using an ApopTag peroxidase Kit (MP Biomedicals, Illkirch, France). Microscopy analyses were performed on a Nikon Eclipse Ti fluorescence microscope (Amsterdam, The Netherlands) or on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany), using the appropriate settings. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, NL).

***Western blotting***

Immunoblotting was performed by homogenizing tissue samples in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using a Bradford protein assay (BioRad, Hercules, CA, USA). Aliquots corresponding to 10 μg of proteins were separated by SDS-PAGE electrophoresis. Blotting and chemiluminescent detection of immunoreactive bands were performed using the V3 Western Workflow system (BioRad, Hercules, CA, USA), according to the manufacturer protocols. Primary antibodies rabbit anti-amylase (Sigma-Aldrich, Buchs, Switzerland), rabbit anti-LC3B (Cell Signaling, Danvers, USA), and rabbit anti GAPDH (Sigma-Aldrich, Buchs, Switzerland) were incubated overnight at 4$^{o}$C.

***Transcript analysis***

The following Taqman probes (Applied Biosystems) were used: Sox9 Mm00448840_m1, Hes1 Mm01342805_m1, Notch-1 Mm00435249_m1, Mist1 (Bhlhb8) Mm00487695_m1, Cpa1 Mm01137017_m1, MCP1/CCL2 Mm00441242_m1, PTF1a Mm00479622_m1, Amy Mm00651524_m1, Aldh1a1 Mm00657317_m1, Hnf1b Mm00447459_m1, Hnf6 (ONECUT1)

Mm00839394_m1, Krt19 Mm00492980_m1, Cckbr Mm00432329_m1, CD146 (Mcam) Mm00522397_m1, Htr2a Mm00555764_m1, Htr2b Mm00434123_m1, SERT (Slc6a4) Mm00439391_m1, Mao A Mm00558004_m1, Mgl1 (Clec 10a) Mm00546124_m1, Nos2 Mm00440502_m1, Mrc1 (MMR, Clec 13D) Mm00485148_m1, Mmp7 Mm00487724_m1, Pdx1 Mm00435565_m1, VEGFα Mm00437304_m1, IL-1b Mm00434228_m1, IL-6 Mm00446190_m1, TNFα (17) Mm00443258_m1, Htr1a Mm00434106_s1, Htr1b Mm00439377_s1, Amy2 Rn00821330_g1, Ptf1a Rn00588645_m1, Hes1 Rn00577566_m1, Sox9 Rn01751069_m1,TaqMan Ribosomal RNA Cont Reagents VIC (18S).

***Statistical analyses***

Groups of 5-6 animals were tested for each experiment. The data are expressed as the means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test unless otherwise stated. A p value < 0.05 was considered to be

significant.

**Figure legends**

**Figure S1**. **A**. Immunostaining of Ki67, 5-bromo-2'-deoxyuridine (BrdU) and phospho-histone 3 (pH3) at the indicated time points following cerulein treatment. Pictures represent an inset (1:10) of the original images used for quantification of positive cell number. **B**. Acinar cells (Ac) positive for Ki67 were identified by large and round nuclei located inside the acinar periphery, while interstitial cells (Int) had smaller and elongated nuclei present outside the pancreatic acini. PU.1 staining confirmed that part of interstitial cells with elongated nuclei was of inflammatory origin. Limited Ki67-αsmooth muscle actin (αSma) co-staining showed that interstitial activated stellate cells were not highly replicative both in WT and TPH1$^{-/-}$ mice. Scale bars: 50 μm.

**Figure S2**. β-catenin, Sox9 and αsmooth muscle actin (αSma) immunostaining seven days after induction of pancreatitis. Nuclei are stained with DAPI (blue). Scale bars: 50 μm.

**Figure S3**. qPCR of genes typical of the differentiated state showed similar expression in the two strains eight hours after cerulein treatment. Cpa1, carboxypeptidase 1. Results are average ± SEM (n=5).

**Figure S4**. **A**. Immunoblotting and quantification of processed LC3 (LC3-II) one day after cerulein treatment. Data are expressed as percentage of total LC3 (I+II). Results are average ± SEM (n=4). **B**. Immunostaining of the autophagic marker p62 at the indicated time points following cerulein treatment. **C**. Quantification of p62 positive areas one day after cerulein treatment. Data are expressed as percentage of the pancreatic area in the field. Scale bars: 50 μm.

**Figure S5**. **A**. Scheme depicting the treatment regimen with 5-HTP and cerulein (Cer). 0.9% NaCl was used as vehicle control. **B**. Quantification of the replication markers Ki67 and pH3 expressed in pancreatic acinar (Ac) and interstitial (Int) cells after three days of 5-HTP supplementation and pancreatitis induction. **C**. Quantification of PU.1 expressing cells infiltrating the pancreas after three days of 5-HTP supplementation and pancreatitis induction. **D**. qPCR of acinar, ductal and progenitor markers after three days of 5-HTP supplementation and pancreatitis induction. **E**. qPCR of inflammation markers after three days of 5-HTP supplementation and pancreatitis induction. **F**. qPCR of inflammation and fibrosis markers after 14 days of 5-HTP supplementation and pancreatitis induction. **G**. Quantification of the replication marker Ki67 expressed in pancreatic acinar cells after 14 days of 5-HTP supplementation and pancreatitis induction. **H**. Quantification of ADM lesions after 14 days of 5-HTP supplementation and pancreatitis induction. **I**. Quantification of PU.1 expressing cells infiltrating the pancreas 14 days of 5-HTP supplementation and pancreatitis induction. Results are average ± SEM (n=5), *p<0.05.

**Figure S6**. **A**. PU.1 immunostaining in WT and TPH1$^{-/-}$ pancreata three days after pancreatitis induction. **B**. qPCR of macrophage markers during the course of pancreatitis induction. **C**. qPCR of TNFα during the course of pancreatitis induction. **D**. qPCR of vascular related factors during the course of pancreatitis induction. Results are average ± SEM (n=5), *p<0.05. Scale bars: 50 μm.

**Figure S7**. **A**. Pan-leukocyte immunostaining with anti-coronin-1 antibody two weeks after PPX. **B** TUNEL staining showed comparable number of apoptotic cells in intact acini in both strains at the indicated times following PPX. **C**. Sirius red staining showed comparable collagen deposition in the two strains two weeks after PPX. Scale bars: 50 μm.

**Figure S8**. **A**. qPCR of 5-HT receptors and transporter in WT and TPH1$^{-/-}$ pancreata during the course of pancreatitis. **B**. qPCR of 5-HT receptors and transporter in WT, TPH1$^{-/-}$ and WT supplemented with 5-HTP mice 24 hours following pancreatitis induction. **C**. qPCR of 5-HT receptors and transporter in WT and TPH1$^{-/-}$ pancreata following pancreatectomy. **D**. qPCR of 5-HT receptors and transporter and 5-HT deaminase enzyme monoaminooxidase A

(MaoA) in acini isolated from WT and TPH1$^{-/-}$ mice one day after pancreatitis induction.

**Figure S9**. **A**. Bright field images of AR42J cells incubated for 2 days with dexamethasone. Note as the cells change morphology upon treatment. **B**. Quantification of AR42J cell number following 48 hours incubation with 50 nM dexamethasone (Dexa). Results are average ± SEM (n=3).


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# 7. Discussion

Acinar-to-ductal metaplasia is a common feature of pancreatitis and it may represent the site of the strongest injury, as ADM is highly inflamed and fibrotic compared to the surrounding tissue. As already mentioned, the extent of ADM depends on injury type and duration. A long lasting injury can trigger an extended ADM development, leading to a total atrophy of acinar cells and functional impairment of the tissue. On one hand, ADM represents a pool of progenitor cells required for tissue regeneration and a remarkable plasticity of pancreatic cells on the other hand, it can cause tissue functional impairment and disease exacerbation.
ADM is also important in the case of pancreatic cancer, as it is considered to be a pre-malignant lesion and pathological conditions that stimulate ADM formation can also stimulate the susceptibility of these cells to tumorigenic transformation. Specifically, an activated tumorigenic pressure, together with cell dedifferentiation, can initiate a cell state that promotes cancer formation. Understanding ADM formation can help to identify therapeutic targets not only for pancreatitis but also for pancreatic cancer. Moreover, the capacity of acinar cell for plasticity makes them an attractive target for diabetes therapy where they might be reprogrammed into insulin-producing β-cells for transplantation. These examples underline the significance of ADM and hence it is critical to explain this phenomenon to improve health related issues.

## 7.1 $p21^{WAF1/Cip1}$ limits ADM formation but not acinar cell proliferation

Pancreatic tissue injury results in ADM formation and also increased acinar cell proliferation, as an attempt to regenerate the damaged tissue. Cells always balance mitogenic and anti-mitogenic factors and this balance allows the cells either to remain in a non-proliferative state or to enter the cell cycle and proliferate. An injury shifts this balance by activating the cell cycle in more cells and inducing a higher rate of proliferation. In normal tissue, the cell cycle is restrained by a set of different factors including cyclin-dependent kinase inhibitors like $p21^{Waf1/Cip1}$ (p21). p21 maintains cells in G0 phase when conditions are not optimal for proliferation [34]. During pancreatitis, two events with opposite effects have been observed in acinar cells: p21 up-regulation and increased cell proliferation. Intriguingly, p21 was strongly expressed in acinar cells but not in ADM areas. Keeping in mind the inhibitory function of p21, it was logical to ask the following questions:

- Does p21 restrain acinar cell proliferation during pancreatic regeneration?
- Are low levels of p21 a prerequisite for ADM formation?
- Is there an association between acinar cell proliferation rate and ADM formation?

To address these questions we studied the course of cerulein-induced pancreatitis in p21 KO mice. In this study we demonstrated a higher incidence of ADM formation in the absence of p21. Moreover, we confirmed p21 involvement in ADM formation *in vitro* by showing an inverse correlation between up-regulation of p21 and reduction of ADM incidence. However, our analysis showed that p21 is a weak suppressor of mitotic activity in both acinar and ADM cells. Despite the fact that the cell replication rate did not increase in the absence of p21, our results showed that p21 can regulate the cell cycle by supressing the expression of cell cycle regulators, for instance cyclins. Taking into consideration these results, it is fair to state that p21 contributes to the inhibition of the cell cycle, however it lacks a full control of mitosis.

Tight control of the cell cycle in acinar cells is an aspect that needs broader investigations. One possible explanation of why we did not detect an increased cell proliferation in p21 KO mice is a compensatory control of the cell cycle achieved by inducing the expression of other cell cycle inhibitors. Indeed, our results showed induction of other cyclin-dependent kinase inhibitors in the absence of p21. It is also reasonable to hypothesise that p21 KO acinar cells, with an activated but de-regulated cell cycle, might dedifferentiate and contribute to metaplasia formation instead of replicating. Thus p21 may act as a tumour-suppressor in the pancreas.

The increased incidence of ADM formation might arise from different mechanisms. One possibility might be an increased acinar cell susceptibility to injury in the absence of p21, however, we did not observed increased cellular damage in p21 KO mice. Another possibility for increased ADM formation might be an altered functionality of non-acinar cells, for example immune or pancreatic stellate cells, which can trigger ADM by producing pro- or anti-inflammatory molecules. We did not investigate in detail the functionality of non-acinar cells, however, our analysis showed similar levels of stellate cell activation but increased levels of pro-inflammatory molecules in p21 KO mice. Finally, increased ADM may result from the increased senescence observed in the absence of p21. Cellular senesce is an irreversible arrest of cell growth induced when cells encounter any kind of stress [35]. In the course of pancreatitis, p21 might act as a molecular switch to promote quiescence of acinar cells and to limit activation of senescence. How can senescence contribute to ADM formation? Interestingly, senescent cells secrete pro-inflammatory cytokines and proteases that promote a microenviroment suitable for ADM development [35]. In support of this hypothesis, our results showed increased levels of these molecules that can contribute to ADM development. A key player mediating the increased senescence and ADM formation is likely to be β-catenin, the expression of which was stronger in p21 KO mice. This molecule has been shown to induce ADM and also mediate senescence and senescence-associated secretory phenotype in cancer [27, 36, 37].

All in all, our findings are consistent with a critical role of p21 in restraining senescence and ADM formation in acinar cells. In addition, we showed that p21 has a limited activity in restraining acinar cell proliferation.

## 7.2 TGF-β limits ADM formation and acinar cell proliferation

TGF-β signalling plays an important role in tissue repair. TGF-β receptor II is the main receptor in TGF-β signalling pathway that links TGF-β ligand binding to downstream activation of intracellular Smads, eventually leading to target gene transcription [37].

Many components of the TGF-β signalling, including three ligands (TGF-β1,2,3) and two receptors (TGF-β RI and RII), are activated during pancreatitis, indicating the significance of this pathway in the disease. Regarding TGF-β function in acinar cells, studies have already shown its' anti-proliferative role in *in vitro* model systems and *in vivo* uninjured pancreatic tissue [38, 39]. However, the anti-proliferative role of TGF-β in pancreatitis and more importantly, during the disease progression has not been investigated yet. In addition, the role of this cytokine in acinar cell dedifferentiation has not been fully established. A higher incidence of ductal structure formation and increased acinar cell proliferation was demonstrated in a single study analysing the effects of abrogated TGF-β signalling in normal pancreatic tissue [38]. However, this study did not investigate any potential mechanisms promoting the displayed phenotype. To address the question whether TGF-β restricts acinar cell proliferation and ADM formation we knocked out TGF-β receptor II (TGF-β RII KO) specifically in pancreatic epithelial cells.

Our study showed no TGF-β interference with the basal mitotic activity of acinar cells in the uninjured pancreas, suggesting that this signalling molecule is not required for homeostatic turnover of acinar cells. Of note, this result differs from the one obtained in a study using a dominant negative form of TGF-β RII, where an increased acinar cell proliferation in the uninjured pancreas was observed [38]. Interestingly, TGF-β also did not interfere with the acinar cell cycle in the early onset of pancreatitis, but elicited a suppressor role of acinar cell proliferation when the severity of the disease advanced. In addition, the increased number of acinar cells positive for the general cell cycle activation marker Ki67 but not for the mitosis marker pH3 observed in TGF-β RII KO mice indicates that TGF-β regulates the initial cell cycle phase but not the final transition to mitosis. The observed down-regulation of cyclin-dependent kinase inhibitor p16 in the absence of TGF-β RII might be the reason for the increased activation of the cell cycle. However, other cell cycle inhibitors can compensate the down-regulation of p16 and further limit the cell cycle progression and in this way preventing completion of the mitosis.

The core result of our study showed that TGF-β is potent enough to reduce the incidence of ADM formation. In the absence of TGF-β RII a higher incidence of ADM formation was observed together with higher expression of EGF receptor, which is a well-known ADM inducer. Thus, a possible crosstalk between TGF-β/EGFR, where TGF-β inhibits the activation of EGFR and acinar cell dedifferentiation, would explain how TGF-β limits ADM formation. In addition, the deregulation of EGFR and p16 expression due to impaired TGF-β RII signalling observed during pancreatitis resembles what observed in pancreatic cancer. During malignant transformation, pancreatic cells lose the function of TGF-β, TGF-β RII and p16 genes and significantly increase the expression of EGFR. Thus, alterations of these genes are critical not only for the formation of ADM but also for pancreatic tumorigenesis.

One would expect that accumulating pancreatic damage during the progression of pancreatitis would be another possible reason for ADM formation. However, we could not detect any higher cellular or inflammatory damage in late stages of pancreatitis suggesting that these processes are independent of each other. However, other studies are required in our TGF-β RII KO mouse model to demonstrate ADM independence from inflammation.

While investigating the TGF-β role in ADM formation, we cannot dismiss a possible TGF-β crosstalk with other signalling pathways. However, the analysis of Akt signalling pathway and various growth factors, which can contribute for ADM expansion, for instance EGF, HGF and TGFα , did not reveal any alteration in gene expression following TGF-β RII ablation. To investigate more thoroughly whether TGF-β interacts with other pathways like autophagy, further analyses of our mouse model are necessary.

Collectively, our study highlighted a crucial role of TGF-β in two processes of pancreatic regeneration. On one hand TGF-β limits acinar cell replication, on the other hand, it acts as a tumour suppressor restricting cell dedifferentiation and metaplasia formation.

### 7.3 Serotonin regulates acinar cell dedifferentiation and ADM formation

As described above, pancreatic regeneration following pancreatitis is characterised by proliferation of pancreatic acinar cells and transient formation of ADM. Previously we investigated the roles of two anti-mitogenic factors in acinar cell proliferation and dedifferentiation. In addition, we were also interested in the role of pro-mitogenic factors in these processes.

Serotonin, a bioactive molecule stored in and released by platelets, induces a mitogenic effect on a wide range of cells, both *in vivo* and *in vitro.* Serotonin triggers proliferation of lymphocytes, hepatocytes, vascular and smooth muscle cells. Furthermore, it also regulates the development of certain organs, for instance brain and mammary gland. Some studies

have shown that hypothalamic serotonin has a functional role in pancreatic regeneration via sympathetic regulation [40]. However, so far no one addressed the question of a direct role of serotonin in pancreatic regeneration. To investigate the mitogenic effects of serotonin in acinar cells we used two regeneration models: partial tissue loss following 60% pancreatectomy and inflammatory damage following cerulein-induced pancreatitis. We compared two types of regenerative stimuli because the pancreatic capacity to regenerate differs according to the severity of tissue damage.

While serotonin did not have any effect on acinar cell proliferation and ADM formation after pancreatectomy, we demonstrated that it was required for ADM formation during pancreatitis. Serotonin necessity for acinar cell dedifferentiation is surprising and can be discussed from different perspectives. At first, due to the known mitogenic function of serotonin, one could expect elevated acinar cell proliferation and in turn increased ADM formation as a consequence of active cell cycling. However, in the absence of serotonin we observed increased acinar Ki67 labelling and higher number of acinar cells in M phase than in G2 phase, while the levels of BrdU and phospho-histone 3 labelling were comparable in the two mouse strains. One explanation for this data can be that the absence of serotonin results in delayed cell cycle progression and accumulation of cells in the early phases of the cell cycle, which would comply with a mitogenic role of serotonin in pancreatic acinar cells. On the other hand, serotonin may elicit an anti-mitotic function in the early phases of the cell cycle, so in its absence more acinar cells can enter but not complete the proliferation process. To clearly define the roles of serotonin in acinar cell proliferation further studies are necessary. However, the fact that acinar replication following pancreatectomy was not affected by the absence of serotonin, indicated not only that serotonin is not a potent mitogen for acinar cells, but also that the requirement of serotonin for ADM formation does not depend exclusively on cell cycle regulation.

Serotonin has been shown to induce the production of pro-inflammatory cytokines [41], which might be the second reason for its requirement in ADM formation. However, analysis of mice treated with the serotonin precursor 5-HTP demonstrated that the immune response and acinar cell dedifferentiation can be uncoupled in the pancreas, because increased acinar cell dedifferentiation correlated with reduced inflammation.

Another cause for serotonin-mediated ADM formation may result from direct stimulation of serotonin receptors or transporter. As mentioned previously, different processes occur during acinar cell dedifferentiation: release of zymogen granules, down-regulation of terminal differentiation genes and up-regulation of progenitor genes. In our study we used transgenic mice with strongly reduced levels of peripheral serotonin but normal levels in nervous system and showed that serotonin regulates pancreatic acinar secretion by stimulating its receptors or transporter in acinar cells and promoting cytoskeletal remodelling. In addition to serotonin-

stimulated release of zymogens, which is necessary for acinar cell dedifferentiation, we also showed that serotonin was required for up-regulation of the progenitor genes Hes1, Sox9 and Notch1. Collectively, our results stress serotonins' role in promoting the progenitor state of acinar cells by stimulating acinar cell secretion and progenitor genes expression, which contribute significantly to ADM development.

## 7.4 Pancreatic and other human epithelial metaplasias

The pancreatic epithelium is one of many epithelial tissues, which forms metaplasia after exposure to damaging factors. Comparative investigation of pancreatic metaplasia with other human epithelial metaplasias might help us identify common features of these pathologies and advance our understanding of their molecular regulation and speed up the discovery of therapies.

All epithelial metaplasias are considered a reversible process resulting in substitution of cells that are able to survive better under circumstances that are harmful for other cells. Epithelial metaplasia is considered an adaptive process resulting in a tissue phenotype change, the underlying molecular mechanisms of which are still not completely understood.

Stratified squamous and glandular epithelium go to metaplasia most often than other types of epithelium and organs like stomach, oesophagus, uterus and bladder frequently contain metaplastic tissue. The most common epithelial metaplasias are intestinal and squamous. Intestinal metaplasia lesions are characterised by the presence of intestinal cell types that occurred in ectopic tissue. [42] The best examples of this type of metaplasia are gastric and Barretts' metaplasia. The first develops due to *H.pylori* infection and the latter due to a gastric reflux [42]. Squamous metaplasia is most common in lungs and urinary bladder. Smoking, asthma and cystic fibrosis are factors predisposing to lung squamous metaplasia formation and vitamin A deficiency, trauma or surgery cause a transformation of urinary bladder epithelium. It is not easy to subgroup pancreatic metaplasia because ADM cells are not fully characterised after transformation. Pancreatic acinar and duct cells are glandular type cells; however, ADM has glandular morphology but does not have secreting function. This definition would imply that pancreatic cell changes follow a glandular-glandular phenotype, however more evidences are necessary to prove this assumption.

Epithelial metaplasias contain some similar features among different organs. First, in many cases they are associated with inflammation occurring in the damage tissue. Second, metaplasia can be initiated when changes occur in the expression of genes that keep a cell identity. [43] Third, metaplasia is proliferative and expanding, however it is also reversible and ADM is not observed after damage resolution. Fourth, various factors and pathways are similarly deregulated in metaplasias of different tissues. Examples of the most commonly

deregulated signalling pathways are Wnt, Notch, Hedgehog, EGFR, Stat3 and NF-κB, however the contribution of each pathway or its factors to a different metaplasia is distinct.

Why is metaplasia important? At first, it is important in the context of regeneration because it generates new cells of the same or different phenotype. Undifferentiated or fully differentiated cell types can be the cell source for regeneration and understanding this process is crucial for the treatment of tissue and organ injuries. A clinical significance of metaplasia is based on two reasons. The most critical is that metaplasia predisposes to the development of cancer. One of the best studied examples is Barretts' metaplasia, which is considered a precursor of oesophageal adenocarcinoma. Understanding the molecular and cellular steps leading to metaplasia could result in the identification of markers suitable for diagnostic or in new therapeutic strategies for disease treatment. The other reason why metaplasia is clinically important is due to its relevance in cell-based therapies to replace damaged cells, tissue or organs. A number of possibilities exist to generate cells for replacement and one of them is to transdifferentiate them directly *in vivo* by adding additional factors [16]. In this case, the research on metaplasia is crucial to help identify those factors. However, while the results on metaplasia and transdifferentiation from the laboratory are encouraging, it is not clear how and when they will be translated to the clinical practice.

# 8. References


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